UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

POSGRADO EN CIENCIAS BIOLÓGICAS

INSTITUTO DE INVESTIGACIONES BIOMÉDICAS

BIOLOGÍA EXPERIMENTAL Y BIOMEDICINA

ANÁLISIS DE POBLACIONES DE *PEROMYSCUS* SP. (RODENTIA: MURIDAE), ASOCIADAS A JALES DENTRO DE LA RESERVA DE LA BIOSFERA SIERRA DE HUAUTLA, MORELOS, MÉXICO: UN ENFOQUE ECOTOXICOGENÓMICO

TESIS

QUE PARA OPTAR POR EL GRADO DE:

DOCTORA EN CIENCIAS

PRESENTA:

M. en C. PATRICIA MUSSALI GALANTE

TUTOR PRINCIPAL DE TESIS: DR. EMILIO ROJAS DEL CASTILLO
INSTITUTO DE INVESTIGACIÓNES BIOMÉDICAS, UNAM
COMITÉ TUTOR: DR. JESÚS JAVIER ESPINOSA AGUIRRE
INSTITUTO DE INVESTIGACIÓNES BIOMÉDICAS, UNAM
DRA. MARÍA ISABEL GARCÍA PELÁEZ
FACULTAD DE MEDICINA, UNAM.

MÉXICO, D.F. ABRIL, 2013

UNAM
POSGRADO
Ciencias Biológicas

COORDINACIÓN

Dr. Isidro Ávila Martínez
Director General de Administración Escolar, UNAM
P r e s e n t e

Me permito informar a usted que en la reunión ordinaria del Comité Académico del Posgrado en Ciencias Biológicas, celebrada el día 11 de febrero de 2013, se aprobó el siguiente jurado para el examen de grado de **DOCTORA EN CIENCIAS** de la alumna **MUSSALI GALANTE PATRICIA** con número de cuenta **95503774** con la tesis titulada **"ANALISIS DE POBLACIONES DE *PEROMYSCUS SP.* (RODENTIA: MURIDAE) ASOCIADAS A JALES DENTRO DE LA RESERVA DE LA BIOSFERA SIERRA DE HUAUTLA, MORELOS, MEXICO: UN ENFOQUE ECOTOXICOGENOMICO"**, realizada bajo la dirección del **Dr. Emilio Rojas Del Castillo.**

Presidente:	DR. DANIEL IGNACIO PIÑERO DALMAU
Vocal:	DRA. SANDRA LUZ GOMEZ ARROYO
Secretario:	DR. JESUS JAVIER ESPINOSA AGUIRRE
Suplente:	DRA. ANA ELENA MENDOZA OCHOA
Suplente:	DRA. MARIA ISABEL GARCIA PELAEZ

Sin otro particular, me es grato enviarle un cordial saludo.

A T E N T A M E N T E
"POR MI RAZA HABLARA EL ESPIRITU"
Cd. Universitaria, D.F., a 06 de Marzo de 2013.

M. del Coro Arizmendi

DRA. MARÍA DEL CORO ARIZMENDI ARRIAGA
COORDINADORA DEL PROGRAMA

AGRADECIMIENTOS

Al posgrado en Ciencias Biológicas, UNAM. Por permitir mi desarrollo profesional y otorgarme todas las facilidades para concluir mi proyecto doctoral.

Al Consejo Nacional de Ciencias y Tecnología (CONACYT). Por el apoyo recibido durante mis estudios (número de becario 102684).

A los miembros del comité tutoral:

Dr. Emilio Rojas del Castillo (Tutor principal): Un infinito y sincero agradecimiento por depositar en mí toda tu confianza, así como las facilidades, la paciencia y la gran libertad otorgada para realizar este proyecto. Tus enseñanzas siempre me acompañarán porque has sido un pilar muy importante en mi vida académica. Gracias por tu amistad y cariño!

Dr. Jesús Javier Espinosa Aguirre: Agradezco muchísimo todos sus acertados comentarios y sugerencias para mejorar el proyecto, además de la disposición y las facilidades para que el proyecto llegara a término. De verdad, muchas gracias!

Dra. María Isabel García Peláez: Por su enorme disposición y toda la confianza depositada en mí y en el proyecto desde sus inicios, además de sus excelentes comentarios y todo el apoyo recibido antes y después de mi doctorado. Has sido muy importante en mi formación académica, muchas gracias!

AGRADECIMIENTOS A TITULO PERSONAL

A los sínodos del examen

Dr. Daniel Ignacio Piñero Dalmau: Muchísimas gracias por la excelente disposición y el interés mostrado en el proyecto. Además sus comentarios siempre sirvieron de guía y reflexión, especialmente para mejorar el proyecto, el examen de candidatura y el escrito de la tesis. Mil gracias!

Dra. Sandra Luz Gómez Arroyo: Gracias por acompañarme siempre en mi desarrollo profesional, por ser un punto cardinal en mi formación y hacer que los logros sean mejores. Le tengo una gran admiración y especial cariño, Gracias por todo!

Dra. Ana Elena Mendoza Ochoa: Agradezco todos sus comentarios y sugerencias para mejorar el escrito de la tesis.

Dr. Efraín Tovar Sánchez: Debo reconocer a quién aunque su nombre no aparezca en ningún papel, ha sido parte fundamental, esencial, e imprescindible en la elaboración de este proyecto. Un eterno agradecimiento e inmenso reconocimiento. Nada de lo que hoy está aquí plasmado hubiera sido posible sin tu amor y dedicación al proyecto. El éxito se alcanza sólo cuando se tiene con quien compartirlo, Gracias!

Al grupo de trabajo del **Dr. Emilio Rojas del Castillo:**

Dra. Mahara Valverde: por sus acertados consejos y sugerencias para que cada una de las etapas de este proceso llegara a buen término.

A **Mónica Martínez:** porque siempre me recibió con un abrazo y nunca faltó la pregunta ¿En qué te ayudo?. Gracias porque siempre estuviste dispuesta a colaborar! Eres mi gran orgullo. Te quiero muchísimo.

A **Alexandra:** a quien con el poco tiempo que tengo de conocerla sé que es una gran persona, excelente científica y sobretodo una alguien que facilita siempre las cosas! Gracias Alex!

A mis compañeros de laboratorio: Paulina, Diego, Mónica, Elia, Nahúm, Martín y Cheros.

Al grupo de trabajo del **Dr. Efraín Tovar Sánchez:**

Dr. Efraín Tovar Sánchez: Trataré de expresar este reconocimiento, aunque debo advertir que no hay uno suficiente, ni expresión que alcance o palabras que pronuncien adecuadamente el agradecimiento que te mereces, todo es insuficiente. Desde el primer día que emprendimos este reto perdí la cuenta de cuantas veces he dicho "gracias". Una vez mas… un eterno agradecimiento por haber estado totalmente involucrado en todos y cada uno de los detalles del proyecto, por resolver todas las dudas académicas y existenciales, por la enorme paciencia, por todo el tiempo robado, por haber contestado todos los días la misma pregunta, "Efra, tengo una duda". Por los cafés mañaneros en el gringo…por ir a todas las salidas, en fin, por hacer de esta historia una de éxito y perseverancia y un recuerdo que nos acompañara toda la vida. "Un esfuerzo total es una victoria completa". Hoy hemos ganado completamente… juntos… una vez más!!

AGRADECIMIENTOS A TITULO PERSONAL

Un agradecimiento muy especial a **Leticia Valencia** quién ha sido mi fiel compañera desde nuestros inicios en el doctorado, si se pudo! Gracias por todos los favores, los ánimos, las buenas platicas y las eternas dudas de los micros!!

A **Elgar Castillo**, te agradezco infinitamente tu excelente disposición para ayudar, colaborar y "estar ahí" para todo lo que alguien pudiera necesitar. De verdad, no sé qué hubiera hecho sin tantas y tantas ocasiones que me ayudaste, desde los pequeños detalles en la compu hasta las buenas discusiones académicas. Te estimo mucho!

A mi queridísima **Guadalupe Rangel** "Lupis". Mi maestra de los micros! Gracias por haber hecho del trabajo de laboratorio algo divertido y alegre. Haciendo los experimentos a tu lado las preocupaciones eran mínimas…tu dedicación y compromiso para los proyectos del laboratorio hacen milagros!! Sobre todo porque siempre encuentras la solución a cualquier problema. Mil gracias Lupis!!

A **Rolando Ramírez**, por su optimismo y confianza en mí. Gracias por los ánimos que me diste que tanto se necesitan durante el doctorado! Te estimo mucho.

A todos aquellos que hicieron posible las colectas de los ratones: **Efra, Edith, Ximena, Efraín y Memo**. Un agradecimiento muy especial a **Don Goyo** quién fue una pieza clave en el desarrollo de este proyecto. Gracias a todos!

A mis **compañeros de laboratorio**: gracias por convertir los días ordinarios en extraordinarios: Lety, Elgar, Rolando, Lupita, Tatiana, Betel, Miguel, Sirius, Javier, Abigail Karen y Wendy.

A **Beatriz Vera**, por toda la ayuda recibida durante estos años, Gracias Bety!

A la M. en C. **Laura Márquez** por todo el apoyo técnico recibido para la realización de la técnica de microsatélites, gracias Laura!

A la colección mastozoológica del CIByC en especial a la **Dra. Elizabeth Arellano y al Dr. Francisco González** por toda la ayuda brindada para la captura e identificación de los ratones.

A la Dra. **Angeluz Olvera** por la ayuda otorgada para la medición de los metales en los tejidos, mil gracias!

Al **laboratorio de Sistemática y evolución del CIByC** por las facilidades concedidas para la realización de los microsatélites.

Al CIByC, UAEM. Por permitir mi estancia en el laboratorio de Sistemática y Evolución durante mis estudios de doctorado.

AGRADECIMIENTOS A TITULO PERSONAL

A dios: por permitirme vivir hasta este momento, con el apoyo de los más preciado para el ser humano: una familia maravillosa!

A mi misma: Por la voluntad de llegar, las ganas de vencer, el deseo de usar todo mi potencial, el coraje de ser rebelde, pero sobre todo por el valor de conquistar mi sueño desde pequeña.

"No vayas adonde te lleve el camino. Ve, en cambio, por donde no hay camino y deja una huella" R. W. Emerson

"Considero más valiente al que conquista sus sueños que al que conquista a sus enemigos, ya que la victoria más placentera es la victoria sobre uno mismo" Aristoteles

A mi esposo, Efraín: por la maravillosa compañía, por el amor más grande, por las sonrisas, por ser un oído atento, un ojo crítico, una boca con elogios sinceros y regaños a tiempo, una mano protectora, una mente brillante, un hombro para descansar, un apoyo en el camino, la energía de cada día, por regalarme tu tiempo. Hace muchos años pensé, "el que fija su mirada en una estrella, nunca cambia de idea". Gracias, te amo y amaré el resto de mi vida.

A mis hijas: Hannah Lizette e Ilana: Gracias por haber sido mis fieles compañeras y la fuente de inspiración necesaria para llegar hasta este día. Gracias por ser el refugio y la fuerza de cada día y una eterna disculpa por todo el tiempo robado. Son mi gran orgullo, los amores más grandes y la verdadera escuela de la vida.

A mi Mamá: por ser un gran ejemplo de grandeza y sabiduría. Mucho de lo que soy y he logrado es gracias a ti. Siento un inmenso amor por ti y es un gran orgullo ser tu hija. Ojalá algún día pueda ser con mis hijas como tú has sido conmigo. Gracias ma, te súper amo.

A mi Papá: Por haberme regalado los momentos más felices de mi vida, por tu amor incondicional, pero sobre todo por el reconocimiento para mí y mi profesión. Sé que ha sido un esfuerzo sin precedentes y por eso puedo decir que eres lo mejor que me ha pasado en la vida. Eres un ser extraordinario, te súper amo. Gracias por todo.

A mis hermanas: Aurora y Rina, las quiero y extraño mucho!!

A mi abuelita Reneé: A este ser digno de admiración….aunque no estés con nosotros físicamente, sé que estas al pendiente de mí, como siempre lo hiciste. Gracias, te amo.

A "Doña Chuya": La queremos y extrañamos mucho. Un fuerte agradecimiento por los años que tuvimos la oportunidad de compartir muchos buenos momentos.

A Adriana González: "Todo mundo quiere tener un amigo, pocos se toman la molestia de ser uno"…. gracias Adriana por tu sincera amistad durante todo este tiempo! Te quiero muchísimo.

DEDICATORIA

CON TODO MI AMOR, CARIÑO Y ETERNO AGRADECIMIENTO

PARA:

EFRAÍN

HANNAH LIZETTE

ILANA

ÍNDICE

RESUMEN

Mussali-Galante, P. 2013. "Análisis de poblaciones de *Peromyscus* sp. (Rodentia: Muridae), asociadas a jales dentro de la Reserva de la Biosfera Sierra de Huautla, Morelos, México: un enfoque ecotoxicogenómico". Tesis de doctorado. Doctorado en Ciencias. Universidad Nacional Autónoma de México.

Los efectos de la contaminación química ambiental se pueden observar en todos los niveles de organización biológica. A nivel de las poblaciones, su estructura y diversidad genética es posible que sean afectadas por la exposición a metales. Este estudio se realizó en Huautla, Morelos, México, un distrito minero donde los principales contaminantes son el plomo y el arsénico. *Peromyscus melanophrys* es un mamífero pequeño que habita en los jales de la región y ha sido considerado como un organismo centinela. Los niveles de bioacumulación de metales se midieron por espectrofotometría acoplada de plasma masas y los análisis genéticos se llevaron a cabo con ocho secuencias cebadoras de microsatélites en 100 individuos de *P. melanophrys* pertenecientes a tres jales y dos sitios testigos. Se analizó el efecto de la bioacumulación de metales sobre parámetros genéticos (diversidad genética individual y poblacional, estructura genética). Se encontró un gradiente de concentración de metales en los tejidos analizados para cada metal y para el índice de bioacumulación. Los mayores valores de diferenciación genética (*Fst* y *Rst*) y el menor número de migrantes por generación (*Nm*) se registraron entre las poblaciones expuestas. Los análisis de distancia genética mostraron que la población más contaminada fue la más distinta genéticamente de las cinco poblaciones estudiadas. Más aún, se registró una relación negativa y significativa entre la diversidad genética (*He, IR*) y la concentración de cada metal y el índice de bioacumulación de metales en *P. melanophrys*. Este estudio demuestra que el estrés ambiental causado por metales, es uno de los factores principales que influye en los niveles y en la distribución de la diversidad genética de las poblaciones de *P. melanophrys* que habitan en los jales de Huautla, Morelos. Se sugiere el uso de cambios en la estructura y diversidad genética de las poblaciones en escalas micro-geográficas como un biomarcador a nivel de la población.

ABSTRACT

Effects of environmental chemical pollution can be observed at all levels of biological organization. At the population level, genetic structure and diversity may be affected by exposure to metal contamination. This study was conducted in Huautla, Morelos, Mexico in a mining district where the main contaminants are lead and arsenic. *Peromyscus melanophrys* is a small mammal species that inhabits Huautla mine tailings and has been considered as a sentinel species. Metal bioaccumulation levels were examined by inductively coupled plasma mass spectrometry and genetic analyses were performed using eight microsatellite loci in 100 *P. melanophrys* individuals from three mine tailings and two control sites. The effect of metal bioaccumulation levels on genetic parameters (population and individual genetic diversity, genetic structure) was analyzed. We found a tissue concentration gradient for each metal and for the bioaccumulation index. The highest values of genetic differentiation (F_{ST} and R_{ST}) and the lowest number of migrants per generation (*Nm*) were registered among the exposed populations. Genetic distance analyses showed that the most polluted population was the most genetically distant among the five populations examined. Moreover, a negative and significant relationship was detected between genetic diversity (*He, IR*) and each metal concentration and for the bioaccumulation index in *P. melanophrys*. This study highlights that metal stress is a major factor affecting the distribution and genetic diversity levels of *P. melanophrys* populations living inside mine tailings. We suggest the use of genetic population changes at micro-geographical scales as a population level biomarker.

INTRODUCCIÓN GENERAL

Presentación

Los estudios que evalúan los efectos de la contaminación ambiental -principalmente por metales- sobre los cambios en la estructura y diversidad genéticas de las poblaciones expuestas son de gran importancia, debido a que generan información sobre los mecanismos que afectan a dichas poblaciones y ayudan a predecir las consecuencias sobre la salud del ecosistema.

Esta investigación contribuye a los estudios ecotoxicológicos que evalúan los cambios microevolutivos de las poblaciones expuestas a metales producto del mal manejo de cierre de minas. Además de robustecer el uso de especies centinelas utilizando biomarcadores a nivel poblacional.

Este estudio se realizó en la localidad de Huautla, Morelos donde se estima que existen alrededor de 780,000 toneladas de residuos mineros (jales), los cuales fueron depositados a cielo abierto y sin ningún cuidado ambiental. Estos jales contienen una gran cantidad de metales que rebasan los límites máximos permisibles, principalmente arsénico y plomo, lo que representa un peligro para la biota circundante, especialmente las poblaciones que residen dentro de los jales de la región. En particular, *Peromyscus melanophrys* es un mamífero pequeño que habita en estos jales y ha estado en contacto directo con los contaminantes de manera crónica.

De acuerdo a lo anterior, la presente tesis está conformada por las siguientes secciones:

PROYECTO DOCTORAL: Esta sección contiene el producto de la tesis en un formato monográfico. El objetivo de esta sección es evaluar la bioacumulación de metales en riñones de individuos pertenecientes a las poblaciones de *Peromyscus melanophrys* asociadas a jales y en los sitios testigo en Morelos, México. Asimismo, se analiza la diversidad y estructura genética de estas mismas poblaciones, mediante microsatélites de ADN. Por último, se determina la densidad poblacional de *P. melanophrys* en los sitios de estudio.

APÉNDICE A: Esta sección presenta el artículo requisito para obtener el grado de doctor en ciencias. "Evidence of population genetic effects in *Peromyscus melanophrys* chronically exposed to mine tailings in Morelos, Mexico". Environmental Science and Pollution Research. 2013. DOI 10.1007/s11356-012-1263-8

APÉNDICE B: Esta sección muestra el artículo de revisión titulado "Biomarkers of exposure for assessing environmental metal pollution: from molecules to ecosystems". Revista Internacional de Contaminación Ambiental. (2013) 29:117-140. Este artículo corresponde al examen escrito de candidatura que se presentó.

APÉNDICE C: Esta sección contiene el artículo titulado "Comparison of two wild rodent species as sentinels of environmental contamination by mine tailings" Environmental Science and Pollution Research (2012) 19:1677–1686, el cual se realizó como actividad complementaria

(participación en la elaboración de un artículo científico), y contiene los antecedentes sobre bioacumulación de metales y daño genético en mamíferos pequeños que habitan los jales de Huautla, Morelos.

APÉNDICE D: Este apartado contiene el artículo de revisión titulado: "Genetic Structure and Diversity of Animal Populations Exposed to Metal Pollution". Reviews Environmental Contamination and Toxicology, el cual se realizó como actividad complementaria (elaboración de un artículo de revisión). El objetivo de esta actividad fue hacer una actualización bibliográfica sobre el efecto de la contaminación ambiental sobre los cambios en la estructura y diversidad genética de poblaciones animales silvestres expuestas a metales.

Problemática ambiental generada por residuos mineros

La gran diversidad y abundancia de los recursos minerales han posicionado a México como una potencia minera. Actualmente, las 32 entidades federativas registran yacimientos mineros y a nivel mundial, destacamos en la producción de oro (Au), plata (Ag), plomo (Pb), cobre (Cu), zinc (Zn) y fierro (Fe). México ocupa el primer lugar a nivel mundial en la producción de plata, quinto en plomo y zinc y onceavo en cobre (INEGI 2011). Sin embargo, como resultado de esta actividad, se han generado una gran cantidad de residuos mineros a lo largo del país, alcanzando el 65% de los residuos industriales que se producen (Mejía et al. 1999).

El proceso de beneficio de yacimientos minerales sulfurados produce residuos mineros de granulometría fina denominados jales, relaves, colas o "tailings" (Gutiérrez-Ruiz et al. 2007). Los jales son creados durante los procesos de recuperación de los metales a partir de minerales metalíferos, después de moler las rocas que los contienen y mezclar las partículas que se forman con agua y sustancias químicas que facilitan la liberación de los metales (Vega 1999; Marin-Guirao et al. 2005).

Los jales que se generan en el proceso de la concentración de minerales de plomo, plata, zinc y cobre, generalmente contienen sulfuros metálicos residuales como la pirita (FeS2), pirrotita (FeS), galena (PbS), esfalerita (ZnS), calcopirita (CuFeS2) y arsenopirita (FeAsS) que son la fuente principal de elementos potencialmente tóxicos (EPT´s). Generalmente, los EPT's son metales como el arsénico (As), cadmio (Cd), plomo (Pb), cobre (Cu), zinc (Zn), hierro (Fe), mercurio (Hg), níquel (Ni), plata (Hg), cromo (Cr), entre otros (Romero et al. 2007). En condiciones normales de operación de jales mineros, y como consecuencia de tormentas y derrames, o bien, por un manejo inadecuado de éstos, puede ocurrir la dispersión de los mismos hacia su entorno. En las zonas áridas es provocada por el viento (eólica) (Moncur et al. 2004; Romero et al. 2008), mientras que en las zonas lluviosas es a través de los escurrimientos superficiales (hídrica) y generalmente se relaciona con la generación de drenaje ácido. Éste se forma por la oxidación de los sulfuros metálicos y se caracteriza por tener valores bajos de pH y altas concentraciones de EPT´s disueltos (Roussel et al. 2000; Moncur et al. 2004). Sin embargo, aunque los jales no sean generadores de drenaje ácido, cuando son abandonados sin implementar controles ambientales, su erosión y lixiviación también pueden causar su dispersión al ambiente con la consecuente contaminación de los recursos naturales (suelos, sedimentos, aguas

superficiales, subterráneas y aire) debido a las altas concentraciones totales de metales pesados contenidos en estos residuos (Gutiérrez-Ruiz et al. 2007). Dada la alta toxicidad asociada a la exposición a metales provenientes de residuos mineros, en un amplio número de especies animales y vegetales, se han documentado efectos en todos los niveles de organización biológica, desde las células hasta los ecosistemas (Legadic et al. 1994; Peakall 1994; Marín-Guirao et al. 2005; Mussali-Galante et al. 2013).

En 2001, Ramírez estimó que en la República Mexicana existen más de 80 jales en operación (Romero y Gutiérrez 2010). Sin embargo, no hay un inventario de la cantidad y situación de los jales inactivos o abandonados. Recientemente, fue aprobada por la secretaría de Medio Ambiente y Recursos Naturales (SEMARNAT 2004) una norma para la adecuada disposición de jales, la cual sólo aplica para proyectos iniciados después de 2004. Sin embargo, como resultado de la actividad minera pasada existen cientos de millones de toneladas de jales dispersos a lo largo de todo el territorio nacional de los que se desconocen sus condiciones de confinamiento o su posible riesgo ambiental (Ramos-Arroyo y Siebe-Grabach 2006).

En México, los estados donde se ha documentado esta problemática son: Hidalgo, Guanajuato, Guerrero, San Luis Potosí, Michoacán, Baja California Sur, Durango, Sonora, Zacatecas, Coahuila, Puebla y Morelos. Específicamente, en este último se encuentran varias regiones mineras abandonadas y una gran cantidad de desechos producidos por esta actividad, de los cuales se desconoce el riesgo ambiental que representan. Un ejemplo de ello lo constituye el poblado de Huautla, ubicado dentro del municipio de Tlaquiltenango (SEMARNAT 2004, 2005).

El caso de Huautla, Morelos

El estado de Morelos, se ha caracterizado por presentar varios distritos mineros que se han explotado por varias décadas. Las minas más comunes han sido de Ag, Pb y Zn, siendo los distritos mineros ubicados en el municipio de Tlaquiltenango los más explotados en el estado.

Durante los siglos XVIII y XIX y hasta 1950 se explotaron seis minas en esta región. Desde 1993 se encuentran cerradas y se localizan dentro de una zona conocida como “Reserva de la Biosfera Sierra de Huautla”, decretada como tal en 1999 (INEGI 2004). Esta reserva

protege cerca de 59,000 hectáreas de selva baja caducifolia, ecosistema que cuenta con una gran biodiversidad de flora y fauna (INEGI 2002).

Dentro de esta zona se encuentra el poblado de Huautla, el cual presenta una riqueza natural en minerales azufrados de Pb y Ag, donde se estima que existen alrededor de 780 mil toneladas de residuos mineros en los cuales los principales contaminantes son el Pb, As, Cd, Zn, y Cu, además de otra cantidad de material no procesado rico en Pb, Cd, Mn y Zn (SEMARNAT 2005). Existen tres jales en la zona los cuales se encuentran a la intemperie y al borde del "*Arroyo Chico"*, el cual se junta con los arroyos *Juchitlán*, *Salitre* y *Atlipa*, para formar el "*Arroyo Grande"* que desemboca en el Río Amacuzac (SEMARNAT 2005), por lo que la lixiviación de estos metales hacia los cuerpos de agua superficiales y subterráneos y su transporte a otras regiones, tiene una gran probabilidad de ocurrir, sobre todo durante la temporada de lluvias. Además, por tratarse de una zona semi-árida la dispersión eólica también influye en su dispersión.

En un estudio realizado por la SEMARNAT, en conjunto con el Instituto Nacional de Ecología (2004, 2005), se determinó que los jales de Huautla contenían elevadas concentraciones de plomo (hasta 3340 mg/kg) y arsénico (hasta 274 mg/kg) rebasando los límites máximos permisibles propuestos por la NOM-127/SSA-1-2004 para As (20 mg/kg suelo residencial y 40 mg/kg suelo industrial) y para Pb (200 mg/kg residencial y 1500 industrial). También se encontraron concentraciones elevadas de cobre, zinc y cadmio. También, se determinó la biodisponibilidad de los contaminantes presentes en las muestras de jales a través de la proporción del metal que puede disolverse (lixiviar) en condiciones ácidas. Se concluyó que de todos los metales analizados, se encontraron solubles As, Cd, Pb y Hg (SEMARNAT 2004, 2005).

Por otra parte, entre los minerales predominantes en la localidad de Huautla, se encuentran: acantita, calcita, calcocita, galena y plata, la cual, además de los minerales anteriores, esta normalmente asociada con cobre, arsénico, cinabrio, cobaltita y barita (The Mineral Database 2004). De esta manera, los altos contenidos de Pb y As detectados en los residuos de la zona, pueden relacionarse directamente con el tipo de minerales explotados.

En relación a la contaminación por metales en la zona y los efectos sobre la salud de las poblaciones humanas y animales que ahí habitan, se han realizado varios estudios. Mussali-Galante (2008); Martínez-Pacheco (2008) y Reyna-Rosas (2009), reportaron elevadas concentraciones de arsénico en el agua que utilizan los pobladores para consumo, (9 veces por arriba de la norma nacional y 22 veces por arriba de la norma internacional). También reportaron altas concentraciones de As en sangre entera de estos mismos pobladores (60 μg/L), con respecto a los pobladores del sitio testigo, donde no se detectó al metaloide. Asimismo, estos mismos autores observaron niveles elevados de daño genético expresado como aberraciones cromosómicas, rompimientos de cadena sencilla de ADN en linfocitos de sangre periférica y micronúcleos en epitelio bucal con respecto a la población testigo.

Con respecto a las poblaciones animales que habitan en los jales de Huautla, se analizó la concentración de metales (Zn, Ni, Fe, y Mn) en hueso e hígado de dos especies de roedores (*Peromyscus melanoprhys* y *Biomys musculus*). En general, se encontraron incrementados todos los metales analizados en ambas especies con respecto a los roedores del sitio testigo. Además se registró daño genético (rompimientos de cadena sencilla) elevado en ambas especies estudiadas con respecto al sitio testigo (Tovar-Sánchez et al. 2012).

Las evidencias antes mencionadas, demuestran que los recursos naturales del sitio de Huautla, Morelos están contaminados por diversos metales provenientes de los jales de la región, lo cual ha afectado a la población humana y a la biota circundante, lo cual compromete la salud del ecosistema (Bickham et al. 2000).

Toxicidad de metales en sistemas biológicos

Biodisponibilidad: dependiendo de los cambios físico-químicos que sufren los metales en el ambiente, éstos se pueden lixiviar, transportar y dispersar al entorno. Es decir, durante la explotación de los minerales, los metales son transferidos a los suelos, pudiéndose acumular localmente en algunas zonas y ser absorbidos por los organismos. Así, diferentes minerales fácilmente alterables como aquéllos que se encuentran en las rocas ígneas y metamórficas, transfieren al suelo cantidades importantes de Mn, Co, Ni, Cu y Zn; la alteración de rocas

ultrabásicas, libera grades cantidades de Cr y Ni, y en menor medida Co (Diez-Lázaro et al. 2002).

Los metales se distribuyen en el suelo en fracciones sólidas con distinto grado de estabilidad: intercambiables, ligados a materia orgánica, a óxidos de hierro y manganeso y a estructuras minerales (González-Flores 2011). El equilibrio dinámico que se establece entre estas fracciones determina su movilidad y biodisponibilidad, siendo el pH, el Eh, la cantidad y tipo de coloides del suelo (materia orgánica, arcillas y óxidos) los factores edáficos más importantes en su control (Weng et al. 2001; Diez-Lázaro et al. 2002). Por lo anterior, la disponibilidad y solubilidad de los metales depende de las reacciones químicas entre éstos y los componentes sólidos del suelo (Basta et al. 2005). Los metales que se encuentran en formas biodisponibles (fracción intercambiable, unidos a arcillas, a materia orgánica u óxidos con enlaces débiles, etc.) son fácilmente incorporados al ecosistema. En cambio, aquéllos unidos a ligandos orgánicos o a cristales son difícilmente separados e incorporados al ecosistema (Kim et al. 2005). La determinación de la concentración de metales en las diferentes fases sólidas presentes en el suelo es útil para conocer su distribución y predecir su comportamiento, lo cual incluye la solubilidad, la movilidad, la biodisponibilidad y por lo tanto la toxicidad (González-Flores 2011).

Bioacumulación: una vez movilizados los metales, pueden ingresar a los organismos y acumularse en ellos, fenómeno conocido como bioacumulación. La bioacumulación es el aumento de la concentración del contaminante en un organismo en cierto tiempo, de forma que su concentración es mayor dentro del organismo que fuera de él (Moriarty 1990). La acumulación de metales en los organismos es un proceso complejo, el cual depende de gran variedad de factores tanto internos (inherentes al organismo) como externos (inherentes al metal) (Gutiérrez-Galindo et al. 1999). Los factores internos que juegan un papel determinante en la acumulación de los metales son: la talla de los organismos (Moriarty 1990), el género (Gutiérrez-Galindo et al. 1999), factores genéticos (Fratini et al. 2008), características de historia de vida, como: ciclos de desove (que afectan la condición y peso de los organismos) (Dauwe et al. 2004) y los hábitos alimenticios (Laurinolli y Bendell-Young 2006). De los factores externos se pueden mencionar: biodisponibilidad del metal, salinidad, pH, temperatura, humedad, naturaleza química, entre otros (Lares y Orianz 1997; Gutiérrez-Galindo et al. 1999; Guerrero 2004).

Naturalmente, el factor más importante es la naturaleza química del contaminante, pues de ella dependen sus características físico-químicas y, de éstas, su presencia, movilidad en el ambiente y su capacidad para interactuar con los organismos e incorporarse a las cadenas tróficas (Albert 2004).

Mecanismos de toxicidad celular: algunos metales se consideran elementos traza o esenciales para los seres vivos, como: sodio (Na), potasio (K) y calcio (Ca), los cuales juegan papeles importantes dentro de las células. Sin embargo, algunos de ellos como el fierro (Fe), cobre (Cu), zinc (Zn), cobalto (Co), molibdeno (Mo) y manganeso (Mn), aunque son esenciales pueden ser tóxicos en altas concentraciones. Otros metales como el mercurio (Hg), plomo (Pb), níquel (Ni), cromo (Cr), cadmio (Cd) y arsénico (As) no son requeridos para actividades metabólicas, por lo que no son esenciales y son tóxicos a concentraciones bajas (Valavandis y Vlachogianni 2010). Por lo anterior, la toxicidad de metales ha sido ampliamente estudiada, donde se ha reconocido que la relación entre la exposición y los subsecuentes efectos sobre la salud son un proceso de varias etapas, que incluye: la exposición externa, la dosis interna, los efectos biológicos tempranos, alteraciones en estructura y función celular, cambios fisiológicos y la aparición de la enfermedad (Link et al. 1995; Vanden- Heuvel y Davis 1999). En este contexto, varios metales son bien conocidos debido a su potencial carcinogénico, como el Cr, Cd, As, Ni y Co (Hartwig 2000). Más aún, estos efectos se extienden más allá del nivel individual, originando alteraciones a nivel poblacional, perturbando la estructura de las comunidades y afectando la salud del ecosistema (Mussali Galante et al. 2013).

Los mecanismos generales de toxicidad de los metales dependen de cuatro factores principales, a) especie química del compuesto metálico, b) duración y tipo de exposición (laboral o ambiental), c) concentración del compuesto(s) y d) recipiente biológico o tipo de organismo expuesto.

En general, los metales tienen la capacidad de evadir barreras naturales de protección del cuerpo y utilizar sistemas fisiológicos normales para afectar diversos constituyentes celulares. Existen dos mecanismos generales por medio de los cuales los metales pueden causar daño celular. El primero involucra la unión del metal con distintas macromoléculas, lo que puede

derivar en un cambio conformacional o bien, éste puede remplazar a metales esenciales de sus sitios activos o sitios de unión y así alterar la homeostasis celular, estos efectos se deben a la gran similitud química de los metales con cationes divalentes esenciales como Zn, Ca, Fe, Mg, entre otros (Leonard et al. 2004). El segundo mecanismo involucra su actividad como centros catalíticos en reacciones tipo redox, las cuales producen especies reactivas de oxígeno (EROs), las EROs son intermediarios que se forman durante los procesos de oxidación metabólica. Las EROs incluyen al anión superóxido ($O_2.^-$), el radical hidroxilo (.OH) y el peróxido de hidrógeno (H_2O_2) (Valko et al. 2005). Las células se pueden adaptar a concentraciones fisiológicas de EROs mediante los sistemas antioxidantes. Sin embargo, altas concentraciones de EROs pueden causar daño oxidante a diversas proteínas, lípidos y sobre todo al ADN. Ambos mecanismos pueden alterar la señalización celular, dando como resultado la alteración del ciclo celular, entre otros procesos (Qian et al. 2003; Valko et al. 2006).

Debido a lo anterior, la mayoría de los metales pueden provocar daño indirecto a la molécula de ADN, causando rompimientos de cadena sencilla y doble (RCS, RCD) (Valko et al. 2006; Mussali-Galante et al. 2007; Frenzilli et al. 2009). También se ha documentado que los metales son capaces de generar aberraciones cromosómicas, intercambios de cromátidas hermanas, formación de micronúcleos, oxidación y alquilación de bases nitrogenadas, entre otros (Fenech et al. 1999; Wilson y Thompson 2007; Rojas 2009; Valavanidis et al. 2009). Asimismo, se ha demostrado que la alteración de las enzimas involucradas en la reparación del material genético es un mecanismo común de toxicidad de diversos metales (As, Cd, Co, Ni), generalmente por el intercambio de iones metálicos esenciales como el Zn, Mn, Ni y Co que generalmente se encuentran en los centros catalíticos de este tipo de enzimas (Hartwig et al. 2002; Rossman 2003).

Efecto de la contaminación ambiental por metales sobre las poblaciones animales expuestas

Genética de poblaciones: la genética de poblaciones es la ciencia que nace de la aplicación del conocimiento mendeliano a las poblaciones, constituye un conjunto de teorías que permiten estudiar la presencia y dinámica de la variabilidad natural de las poblaciones, así como su

significado evolutivo, por lo que su historia está íntimamente ligada al descubrimiento y formalización de la teoría evolutiva (Fontdevilla y Moya 1999).

La teoría de genética de poblaciones explica que la variación genética se puede descomponer en diferentes factores (Piñero 2008). Entre éstos, se encuentra el de estructura genética, el cual se define como el arreglo no aleatorio de las frecuencias alélicas y genotípicas en las poblaciones y se constituye por la manera en cómo se distribuye la diversidad genética dentro y entre las poblaciones (Loveless y Hamrick 1984; Coutellec y Barata 2011).

El conocimiento de la estructura y diversidad genética es fundamental para entender el origen y la evolución de las poblaciones en condiciones naturales (Hernandez et al. 2006). Sin embargo, su estudio así como el de la genética de poblaciones en general, no solo tiene fuertes implicaciones en la biología evolutiva y ecología, sino que ha llegado a desempeñar un papel muy importante en la conservación de la diversidad genética (Piñero, 2008).

Factores que modifican la estructura y diversidad genética de las poblaciones: la evolución y el mantenimiento de la estructura genética en el tiempo y en el espacio es resultado de la interacción de varias fuerzas evolutivas: a) selección natural, b) deriva génica, c) mutaciones, d) flujo genético (migración) y e) recombinación (Loveless y Hamrick 1984; Coutellec y Barata 2011). Además de lo anterior, la forma en que la variación genética se distribuye dentro y entre poblaciones está determinada por factores ecológicos como: a) la historia de vida de las especies, b) su dispersión, c) disturbios y fragmentación del hábitat (Barret y Khon 1991). Existen factores evolutivos que pueden incrementar o disminuir la diversidad genética de las poblaciones. Dentro del primer grupo, se puede citar a las mutaciones y al flujo genético, mientras que dentro del segundo grupo es posible mencionar a la deriva génica (Dobzhansky 1993). Estos factores influyen directamente sobre la estructura genética, ya que ésta depende en gran medida de la cantidad de variación genética que se reparte dentro y entre las poblaciones

Se ha reportado que la estructura y diversidad genética de las poblaciones puede verse afectada por diferentes disturbios que causan la fragmentación o pérdida del hábitat, lo que resulta en discontinuidades, con consecuencias negativas para la biota. Específicamente, la fragmentación del hábitat puede alterar la estructura genética por medio de la reducción en el número de individuos de una población y las discontinuidades entre las poblaciones cercanas

puede provocar un flujo genético reducido entre ellas, lo que a su vez, puede incrementar la intensidad de la deriva génica (Peakall y Lindmeyer 2006). Además, si lo anterior se produce en una población de tamaño pequeño, la pérdida de variabilidad genética asociada a un incremento en la endogamia, puede disminuir la capacidad de adaptación de los individuos a cambios ambientales, reduciéndose entonces, las probabilidades de sobrevivencia de la población afectada (Herbert y Murdoch-Lukier 1996; Bickham et al. 2000).

La contaminación ambiental por metales como un factor que modifica la estructura y diversidad genética de las poblaciones: durante la última década, el estudio de los efectos de los metales sobre la estructura y diversidad genética en poblaciones naturales se ha convertido en un tema prioritario en estudios de ecotoxicología (Bickham y Smolen 1994; Gutman 1994; Bickham et al. 2000; Peles et al. 2003).

Aunque los efectos inmediatos de la exposición a metales ocurren a nivel molecular y celular, éstos se pueden extender a niveles de organización mayores. Lo anterior ha sido definido por Bickham y Smolen (1994) como "efectos emergentes"; los autores explican que aunque el daño sea a nivel celular o sub-celular, los efectos emergentes se pueden observar en la población, la comunidad y el ecosistema. Particularmente, las poblaciones, pueden sufrir alteraciones en su diversidad y estructura genética (Bickham et al. 2000). Aunque existen varios factores involucrados en la alteración del reservorio genético de las poblaciones, la contaminación del ambiente, especialmente las exposiciones crónicas a dosis bajas es uno de los factores implicados en los cambios de la diversidad y estructura genética de las poblaciones expuestas a agentes químicos, especialmente si estos últimos tienen la capacidad de dañar al material genético de manera directa o indirecta, como es el caso de algunos metales. Por lo anterior y con el objetivo de analizar estos efectos en poblaciones silvestres, la utilización de biomarcadores a nivel de la población, como los cambios en el reservorio genético de las poblaciones expuestas crónicamente a metales es necesario para establecer una relación entre los contaminantes ambientales y los efectos a nivel de la población.

En este sentido, varios autores (Van Straalen 1999; Van Straalen y Timmermans 2002; Maes et al. 2005) han descrito cuatro mecanismos por medio de los cuales los metales (y otros contaminantes ambientales) pueden alterar la diversidad y estructura genética de las poblaciones animales expuestas: 1) algunos agentes pueden ser genotóxicos, mutagénicos y alterar procesos

de reparación del ADN, lo que puede incrementar la carga mutacional de los individuos; 2) la exposición a los productos tóxicos favorece la permanencia de genotipos tolerantes y/o la eliminación de aquellos intolerantes, cambiando así la composición genética de la población expuesta; 3) los compuestos tóxicos pueden causar cuellos de botella (reducción del tamaño poblacional) y 4) alterar las tasas o patrones de migración de la población, lo que puede aumentar o disminuir el flujo genético entre poblaciones cercanas.

Como resultado de lo anterior, los cambios en la estructura y diversidad genética de las poblaciones expuestas pueden ser utilizados como un biomonitor de la salud del ecosistema. Además, considerando que la diversidad genética es uno de los pilares de la biodiversidad y evolución (Duan et al. 2001; Medina et al. 2007) y especialmente porque la pérdida de diversidad, puede ser permanente (dependiendo del tamaño poblacional y la tasa mutacional), el estudio de cómo la exposición a metales puede derivar en cambios de las frecuencias alélicas y genotípicas de las poblaciones expuestas se torna en una prioridad en biomonitoreos ambientales y programas de conservación.

Respuestas genéticas de las poblaciones silvestres expuestas a metales: las respuestas que ocurren al interior de las poblaciones expuestas a metales pueden tener un profundo efecto sobre su variabilidad y estructura genética. Generalmente, estas respuestas suceden de dos maneras: a) aumento de la variabilidad genética como consecuencia de nuevas mutaciones inducidas por los agentes genotóxicos, o b) disminución de la variabilidad genética como resultado de cuellos de botella o selección (Mussali-Galante et al. 2013). En ambos casos, estas respuestas pueden ser la consecuencia de la adaptación de las poblaciones a ambientes contaminados (Bickham et al. 2000; Berckmoes et al. 2005; Maes et al. 2005; Gardeström et al. 2008; Durrant et al. 2011).

En la última década, se han realizado decenas de estudios los cuales han analizado la estructura y diversidad genética de poblaciones animales silvestres expuestas a metales. Nueve de éstos reportes son artículos de revisión (Bickham et al. 2000; Clements 2000; Belfiore y Anderson, 2001; Staton et al. 2001; Theodorakis, 2001; Van Straalen y Timmermans 2002; Medina et al. 2007; Morgan et al. 2007; Hoffmann y Willi, 2008) y 25 son artículos originales. De éstos últimos, 15 fueron realizados en ecosistemas acuáticos y 11 en terrestres (Para más detalle ver tabla 1, apéndice D).

Dentro de los estudios realizados en ecosistema acuáticos, la mayoría (73.3%) reportaron una disminución de la diversidad genética en las poblaciones expuestas a metales con respecto a las poblaciones testigo, independientemente del tipo de exposición (mezclas o un solo un tipo de metal). La mayoría de las especies estudiadas estuvieron expuestas a una mezcla de metales, como: *Leander intermedius* y *Platynympha longicuadata:* Cd, Zn, Cu, Pb, Mn (Ross et al. 2002); *Littorina brevicula:* Cd, Zn, Cu, Pb, (Kim et al. 2003); *Anguilla anguilla*: Cd, Zn, Pb, Hg, Ni, Cr, As, Se (Maes et al. 2005); *Attheyella crassa*: Zn, Cu, Pb, Hg (Garderstrom et al. 2008); *Perca flavescens*: Cd, Cu (Bourret et al. 2008). Las especies que estuvieron expuestas a un solo metal, fueron: *Pleurocerca canaliculatum*: Hg, (Benton et al. 2002); *Heterandria formosa*: Cd (Athrey et al. 2007); y *Rana ridibunda*: Hg, (Matson et al. 2006). En contraste, no se encontró efecto significativo de la exposición a metales sobre la diversidad genética en cuatro especies expuestas en comparación con los sitios testigo: *Hyalella azteca*: Cd, Zn, (Duan et al. 2001), *Gobio gobio*: Cd, Zn (Bervoets y Blust 2003); *Littorina littorea*: Ag, As, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Zn (De Wolf et al. 2004) y *Salmo trutta*: Cd, Zn (Durrant et al. 2011). Lo cual representó el (26.7%) del total de las especies analizadas.

Por otro lado, en los estudios realizados en ecosistemas terrestres, se analizaron 10 especies distintas. Se reportó que en 40% de éstas hubo una reducción en su diversidad genética con respecto a las poblaciones no expuestas. Algunos ejemplos son: *Cognettia sphagnetorum*: Cu (Haimi et al. 2006); *Talitrus saltator*: Cd, Hg, Cu, (Ungherese et al. 2010); *Pachygrapsus marmoratus:* As, Pb, Cd, Co (Fratini et al. 2008); *Ficedula hypoleuca*: Cd, Zn, Pb, Cu, Ni, Al, As, Cr, Se (Eeva et al. 2006). En contraste, se observó un incremento de la diversidad genética en 40% de las especies analizadas, como: *Lumbricus rubellus*: Cd, Zn, Cu, Pb (Peles et al. 2003); *Capea nemoralis*: Cd, Cr, Cu, Ni, Pb, Zn (Jordaens et al. 2006); *Parus major*: Cd, Zn, Cu, Pb, Ni, Al, As, Cr, Sn (Eeva et al. 2006) y *Larus argentatus* (acero), (Yauk et al. 2000). Mientras que para el 20% restante de las especies, su diversidad genética no se vio afectada por la exposición a metales, como: *Apodemos silvaticus*: Cd, Zn, Cu, Pb, Ni, Al, Ag, As, Co, Mn, Fe (Berckmoes et al. 2005) y *Succinea putris*: Cd, Cr, Cu, Ni, Pb, Zn (Jordaens et al. 2006). En la mayoría de los estudios antes mencionados, la actividad minera o algún proceso relacionado con la minería fue la principal fuente de exposición. Los metales más comúnmente encontrados fueron Cd, Zn, Cu y Pb. Sin embargo, la abundancia de éstos varió entre ecosistemas acuáticos (Cd=Zn>Cu>Pb>Hg) y terrestres (Cu>Cd>Pb>Zn>Ni).

En este contexto, la selección de algunos genotipos tolerantes y la eliminación de algunos genotipos intolerantes, así como reducciones del tamaño poblacional (cuellos de botella) son dos procesos que pueden ocurrir en las poblaciones expuestas a contaminantes ambientales, como los metales. Ambos procesos pueden afectar el potencial adaptativo de las poblaciones expuestas con consecuencias en niveles de organización mayores como la comunidad y el ecosistema (Gillespie y Guttman 1989; Theodorakis et al. 2000; Harper-Arabie et al. 2004; Athrey et al. 2007; Brown et al. 2009) (ver detalles en apéndice D).

Marcadores moleculares para analizar la estructura y diversidad genética de las poblaciones

La aplicación de tecnologías basadas en la secuenciación de ADN y la reacción en cadena de la polimerasa (PCR) en los últimos 20 años, ha permitido la generación de nuevos marcadores moleculares los cuales se han aplicado en diversos campos de la biología (D´Surney et al. 2001).

Los marcadores moleculares son caracteres observables (su expresión indica presencia o ausencia de ciertos genes o secuencias) que juegan un papel importante en la estimación de la diversidad y estructura genética entre individuos, al comparar sus genotipos en un número de loci polimórficos (Arif y Khan 2009). Dentro de los marcadores moleculares que se han utilizado en estudios de ecotoxicología genética, se pueden mencionar los análisis de ADN mitocondrial (ADNmt), ADN nuclear (ADNn), las alozimas, los polimorfismos de longitud de fragmentos de restricción (RFLP´s), los fragmentos amplificados al azar de ADN polimórfico (RAPD´s), los polimorfismos en la longitud de fragmentos amplificados (AFLP´s) y las secuencias simples repetidas o microsatélites (SSR´s), éstos últimos han sido los marcadores más comúnmente utilizados para este tipo de estudios (Cuadro 1) (Para mayor detalle ver apéndice D, tema 5).

Secuencias simples repetidas o microsatélites (SSR´s): para este estudio se eligieron los microsatélites, los cuales se caracterizan por ser secuencias cortas repetidas en tándem, hipervariables de uno hasta cinco pares de bases, los cuales se repiten desde cinco hasta 100 veces en un sitio (Hancock 1999). Los microsatélites están distribuidos azarosamente tanto en el genoma mitocondrial como en el nuclear, su frecuencia de aparición en el genoma es de 1 en cada 6-10 pares de bases (Hancock 1999). Su análisis detecta variaciones en la longitud de los repetidos. Presentan una herencia co-dominante y son de las secuencias que evolucionan más rápido ya que se caracterizan por tener altas tasas de mutación (10^{-2} – 10^{-3} por locus por gameto

por generación). Estos marcadores son considerados como una de las mejores herramientas moleculares para el estudio de la diversidad y estructura genética de las poblaciones (Yauk and Quinn 1996; Athrey et al. 2007; Tremblay et al. 2008) ya que ofrecen varias ventajas: a) alto nivel de polimorfismo debido a su alta tasa de mutación, b) aunque los SSR parecen ser especie-específicos, se pueden utilizar con especies cercanas filogenéticamente, c) se requiere de una pequeña cantidad de ADN, d) alta reproducibilidad, e) ampliamente distribuidos en el genoma (Cuadro 1). Sin embargo, tienen un alto costo ya que se requieren de técnicas de clonación y secuenciación para la generación de secuencias cebadoras específicas.

La abundancia de polimorfismos en los microsatélites se debe a su sistema de mutación, el cual consiste en el apareamiento erróneo durante la replicación, fenómeno conocido como "slippage mispairing". Dicho apareamiento ocurre en las regiones del ADN donde existen pequeñas repeticiones contiguas y es uno de los mecanismos que explica las inserciones y deleciones de repetidos de ADN (Hancock 1999), también expone el hecho de que secuencias de los SSR sean tan conservadas, ya que este mecanismo de mutación no causa alteraciones en las secuencias flanqueantes de los SSR.

Existen dos modelos de mutación principales, el primero se le conoce como "modelo de alelos infinitos" (Kimura y Crow 1964) y el segundo es conocido como "modelos de mutación paso a paso" (Ohta y Kimura 1973).

El primer modelo predice que toda mutación crea un alelo nuevo o que no existía hasta ese momento en la población (Hancock 1999). También supone que el proceso de mutación borra cualquier memoria del estado alélico previo (Slatkin 1985). En el segundo modelo, el proceso de mutación tiene "memoria". Alelos de tamaños similares son menos diferentes que alelos muy distintos en su tamaño (Jarne y Lagoda 1996).

Finalmente, la revisión bibliográfica realizada para este estudio mostró que los SSR´s son los marcadores moleculares que se han utilizado con mayor frecuencia en estudios de ecotoxicología genética (ver apéndice D).

Cuadro 1. Algunas características de los diferentes tipos de marcadores moleculares utilizados en estudios de ecotoxicología genética. SSR's= secuencias simples repetidas/microsatélites. PCR= reacción en cadena de la polimerasa, ADNmt= ADN mitocondrial, ER= enzimas de restricción, RAPD= fragmentos amplificados al azar de ADN polimórfico, RFLP = polimorfismos de longitud de fragmentos de restricción, AFLP= polimorfismos en la longitud de fragmentos amplificados.

Marcador molecular	Descripción	Tipo de herencia	Se requiere información previa de la secuencia	Nivel de polimorfismo
SSR	PCR de secuencias simples repetidas	Co-dominante	Si	Alto
ADNmt	Amplificación por PCR y directamente secuenciadas o cortadas con ER para generar RFLP´s de ADNmt	Co-dominante	Si	Medio-alto
RAPD	Distribución azarosa de secuencias cebadoras en el genoma	Dominante	No	Medio-alto
RFLP	Detección de sitios de restricción	Co-dominante	No	Medio
AFLP	PCR de fragmentos de restricción utilizando secuencias cebadoras modificadas	Co-dominante	No	Medio
Alozimas	Detección de aminoácidos por sus diferencias eléctricas	Co-dominante	No	Bajo

Uso de organismos centinelas para estudios de ecotoxicología genética

Un paso muy importante para poder establecer relaciones entre los efectos de los contaminantes y las respuestas genéticas de las poblaciones expuestas, es el uso de organismos bioindicadores o centinelas. El NRC (National Research Council) ha definido a una especie centinela como "un sistema animal que sirve para identificar riesgos potenciales para otros animales o humanos" (NRC 1991). Claramente, las especies centinelas son de gran utilidad e importancia para estudios de salud ambiental, debido a que proveen información integral tanto de la exposición (información del tipo, concentración y biodisponibilidad de los contaminantes) como del efecto (Basu et al. 2007). Para que una especie pueda considerarse como centinela debe cumplir con los siguientes criterios: a) amplia distribución geográfica, b) habilidad para bioacumular contaminantes, c) fácil captura, d) poca movilidad, e) amplio conocimiento de su biología, f) sensible a los contaminantes, g) ciclo de vida corto y h) mantenimiento en cautiverio (Beeby 2001; Fox 2001; Basu et al. 2007).

En muchos casos, las especies centinelas se utilizan para analizar riesgos para otras especies filogenéticamente cercanas o que puedan ocupar un nicho ecológico similar dentro del ecosistema. En general, muchas especies animales silvestres se han utilizado como centinelas en estudios de ecotoxicología genética de metales, algunas de las cuales son: el pez mosquito (*Gambusia affinis*) (Roark et al. 2001), varias especies de isópodos, copépodos y gasterópodos (Ross et al. 2002; Storelli y Marcotrigiano 2005; Gardeström et al. 2008), lombrices de tierra (*Lumbricus rubellus*) (Peles et al. 2003), varias especies de nemátodos (Ekschmitt y Korthals 2006), el mejillón cebra (*Dreissena polymorpha*) (Sues et al. 1997), el caracol de jardín (*Helix aspersa*) (Nedjoud et al. 2009), varias especies de aves marinas (Burger y Gochfeld 2004), dos especies de cangrejos de mar (*Austropotamobius pallipes* y *Pacifastacus leniusculus*) (Antón et al. 2000), y varias especies de bivalvos (Ma et al. 2000; Ross et al. 2000; Storelli y Marcotrigiano 2005). Con base en los datos anteriores, se puede observar la falta de estudios de ecotoxicología genética de metales en especies de pequeños mamíferos (ver apéndice C).

Los pequeños mamíferos pueden ser de gran utilidad en este tipo de estudios dado que juegan papeles ecológicos importantes. Ocupan una gran variedad de nichos, son recicladores de nutrientes, influyen en las comunidades de plantas e insectos y sirven como presas de numerosos predadores (Levengood y Heske 2008). Además cumplen con varios de los criterios antes

mencionados para ser considerados como organismos centinelas, como: a) amplia distribución geográfica, b) gran abundancia c) poca movilidad (Levengood y Heske 2008), d) hábitos generalistas, e) ciclo de vida corto (Phelps y McBee 2009), f) alta tasa reproductiva y g) facilidad de captura (Laurinolli et al. 2006), lo que los hace un sistema ideal para el estudio de los efectos toxicológicos de los metales (Talmage y Walton 1991; Pascoe et al. 1994; Laurinolli et al. 2006; Levengood y Heske 2008). Más aún, juegan un papel importante dentro de las cadenas tróficas y son considerados como intermediarios en la transferencia de metales desde el suelo hasta niveles tróficos más elevados (Talmage y Walton 1991; Levengood y Heske 2008). También hay evidencia de que estos organismos acumulan metales en diferentes tejidos cuando están cerca de sitios mineros (Anthony y Koslowski 1982; Smith y Rongstad 1982; Beyer et al. 1985; Ma et al. 1991; Beyer y Storm 1995; Levengood y Heske 2008), cerca o dentro de los jales (Cooke et al. 1990; Laurinolli y Bendel-Young 2006) o bien, cuando se encuentran cerca de industrias procesadoras de metales (Jhonson et al. 1978; Kisseberth et al. 1984). Debido a lo anterior, varios efectos han sido documentados en estos organismos, como: teratogénesis, mutagénesis, y alteraciones reproductivas debidas a la exposición crónica a metales (Baranski 1987; Talmage y Walton 1991; Sunderberg y Okarsson 1992; Eisler 1997; Husby et al. 1999; Bisser et al. 2004).

Otra de las ventajas al utilizar pequeños mamíferos como organismos centinelas es el amplio conocimiento de su genoma, lo cual ha permitido desarrollar más de 100 secuencias cebadoras de microsatélites para evaluar parámetros de estructura y diversidad genética (Mullen et al. 2006).

De los pocos estudios que evalúan los efectos de los metales sobre la estructura y diversidad genética de poblaciones silvestres de pequeños mamíferos, especialmente roedores expuestos de manera ambiental, se puede citar el trabajo de Berckmoes et al. (2005) con el ratón de campo (*Apodemus sylvaticus*), Gileva et al. (2008) con el topillo rojo (*Clethrionomys glareolus*) (ver apéndice C).

Por todo lo anterior, utilizar organismos centinelas en estudios de ecotoxicología, facilita el estudio de las relaciones entre la exposición a los metales y las respuestas genéticas en las poblaciones que están en contacto con éstos.

Efectos genéticos en poblaciones de *Peromyscus melanophrys* expuestas crónicamente a jales mineros en Morelos, México.

La actividad minera genera residuos conocidos como "jales" y drenaje ácido, los cuales introducen al ambiente una gran variedad de agentes químicos -como los metales- que contaminan los recursos naturales y la biota circundante (Jiang et al. 2011). Dependiendo de su persistencia y toxicidad, varios metales pueden causar efectos adversos en todos los niveles de organización biológica, ya que éstos han sido identificados dentro de los agentes más tóxicos para casi cualquier organismo (EPA 2000; WHO 2007). Por lo anterior, la exposición ambiental a metales puede afectar a las poblaciones de diferentes formas, una de ellas es la alteración de su reservorio genético (Morgan et al. 2007; Bickham 2011).

Los metales pueden afectar directa o indirectamente el reservorio genético de las poblaciones. Directamente, a través de sus diversos mecanismos de mutagénesis en células somáticas y germinales o indirectamente, a través de procesos ecológicos como alteraciones en la dinámica poblacional, causando cuellos de botella o seleccionando ciertos genotipos resistentes y eliminando aquéllos no resistentes (Berckmoes et al. 2005). Por lo anterior, los estudios de genética de poblaciones permiten analizar y monitorear los impactos ambientales en sistemas naturales (Belfiore y Anderson 1998, 2001; Bickham 2011).

Para poder establecer una relación entre los efectos de los contaminantes y las respuestas a nivel poblacional, la utilización de organismos centinelas es un paso muy importante en estudios de tipo ecotoxicológico, ya que pueden ser utilizados para evaluar la presencia o aumento en el ambiente de uno o varios agentes tóxicos (Rogstad et al. 2003). En este contexto, los mamíferos pequeños son considerados como organismos centinelas debido a su gran abundancia, amplia distribución geográfica, baja dispersión, contacto directo con los contaminantes debido a ingestión e inhalación de suelos contaminados, fácil captura y ciclo de vida corto (Levengood y Heske 2008).

Actualmente, los estudios que analizan la relación entre la exposición a metales y los efectos a nivel poblacional utilizan como marcador molecular las secuencias simples repetidas o microsatélites (SSR's) debido a su alto nivel de polimorfismo, abundancia en el genoma y facilidad de amplificación mediante PCR. Además, debido a su alta variabilidad y rápida evolución han sido

recomendados para estudios que pretenden analizar cambios genéticos recientes entre las poblaciones, tales como los causados por agentes tóxicos ambientales, como los metales (Bickham et al. 2000; Berckmoes et al. 2005; Ariff y Khan 2009).

En particular en el distrito minero de Huautla, Morelos, México, se han reportado 780,000 toneladas de residuos mineros donde los principales contaminantes son el Pb, Mn, Cd y As, los cuales no han sido contenidos o neutralizados (SEMARNAT 2005). Como resultado de lo anterior, hay tres jales en la zona los cuales se encuentran a la intemperie y sin regulación alguna.

Estudios previos en poblaciones animales que habitan en el jale principal de Huautla, documentan la bioacumulación de metales (Zn, Ni, Fe, y Mn) en hueso e hígado de dos especies de roedores (*Peromyscus melanophrys* y *Biomys musculus*). En general, se encontraron incrementados todos los metales analizados en ambas especies con respecto a los roedores del sitio testigo. Además se registró daño genético (rompimientos de cadena sencilla de ADN) elevado en ambas especies estudiadas con respecto al sitio testigo (Tovar-Sánchez et al. 2012).

Las evidencias antes mencionadas, demuestran que los recursos naturales de este sitio están contaminados por diversos metales provenientes de los jales de la región, lo que ha afectado a la biota circundante lo cual pudiera comprometer la salud del ecosistema.

JUSTIFICACIÓN

Existen datos que muestran concentraciones elevadas de diferentes metales en el suelo, agua y jales de Huautla, Morelos, lo cual posiblemente representa un riesgo importante para las poblaciones animales que ahí habitan. Por lo anterior, es importante evaluar los efectos ecotoxicológicos de dicha exposición sobre las poblaciones de *Peromyscus melanophrys* que habitan en los jales de la región.

- Para cumplir con esta necesidad, se requiere evaluar la cantidad y tipo de metales presentes en los individuos expuestos, así como el efecto de los metales sobre la estructura y diversidad genética en organismos centinelas, así se podrá generar

información acerca de los mecanismos de acción involucrados en la afectación de dichas poblaciones.

- Asimismo, es prioritario realizar un estudio que sirva como plataforma para tomar medidas ambientales y reducir los riesgos ecológicos y a la salud de otras poblaciones que se encuentren en situaciones similares.
- Por último, estudios de este tipo, son especialmente importantes ya que ayudan a entender los mecanismos de acción involucrados entre cambios en los biomarcadores y las respuestas ecológicamente relevantes.

OBJETIVO GENERAL

Analizar la estructura y diversidad genética en poblaciones de *Peromyscus melanophrys* asociadas a jales de Huautla, Morelos, México.

OBJETIVOS PARTICULARES

Medir las concentraciones de metales (Al, Pb, Cu, As, Cd, Hg, Ni) en riñones de individuos pertenecientes a las poblaciones de *Peromyscus melanophrys* asociadas a jales y en los sitios testigo en Morelos, México, mediante espectrofotometría de plasma masas.

Analizar la diversidad y estructura genética de poblaciones de *Peromyscus melanophrys* asociados a jales y en los sitios testigo en Morelos, México, mediante microsatélites de ADN.

Analizar la densidad poblacional de *Peromyscus melanophrys* asociados a jales y en los sitios testigo en Morelos, México

HIPÓTESIS

Los jales de Huautla son disturbios que promueven la fragmentación del hábitat, además de contener EPT's biodisponibles, facilitando su incorporación en los tejidos de los individuos expuestos. Por ello, se espera que este tipo de disturbio provoque que las poblaciones de *P. melanophrys* que habitan en los jales, queden en parches y parcialmente aisladas. Además los individuos expuestos pudieran presentar elevadas concentraciones de metales, afectando su salud y reduciendo la densidad poblacional. Como resultado de lo anterior, se espera encontrar una baja diversidad y alta diferenciación genética de las poblaciones expuestas con respecto a las poblaciones testigo.

METODOLOGÍA

Sitios de estudio

En total, se muestrearon cinco sitios ubicados dentro de la REBIOSH, en el municipio de Tlaquiltenango, Morelos. Tres sitios contaminados (jales), ubicados en Huautla, y dos sitios testigo, ubicados en Ajuchitlan y Quilamula (Fig. 1).

Sitios testigo

Ambos sitios testigos se localizan en la parte sur-oeste del municipio de Tlaquiltenango. El testigo l se ubica en las coordenadas 18°27'52″N y 98°58'53″O, y tiene una altitud de 1060 m (INEGI 2004, 2009). El testigo 2 se ubica en las coordenadas 18°30´520″N y 98°59´59″O y presenta una altitud de 1.070 m (INEGI 2004, 2009).

Estos sitios se establecieron como poblaciones testigo del estudio ya que las condiciones geográficas y climáticas son muy similares a las de los sitios expuestos. Además no existen reportes que indiquen contaminación por metales o actividad minera en estas zonas (INEGI 2004, Mussali 2008). En promedio, los sitios testigo se encuentran a una distancia de 7.95 ± 1.53 km de los jales estudiados.

Sitios expuestos

Los sitios expuestos son conocidos por su gran tradición minera. Las minas de esta región explotaron principalmente Pb, Zn y Ag. Durante estas actividades tres jales se formaron en la

zona los cuales fueron generados y manejados de la misma manera por lo que todos ellos tienen una composición química similar.

Jale 1. Es el más pequeño de la zona y se encuentra a 18°25′40.60″N–99°01′57.82″O.

Jale 2. Es el de tamaño intermedio entre el jal1 y el 3 (118 × 92 m.) y se encuentra a 18°26′22.62″ N–99°01′51.71″ O.

Jale 3. Es el más grande de la zona (180 × 80 m.) y se encuentra a 18°26′36.37″N–99°01′26.71″ O. Se localiza a 500 m del poblado de Huautla. Este jal es el más estudiado en cuanto a sus características físico-químicas y contenido de metales. Está rodeado de selva baja caducifolia (Rzedowski 2006), los valores de pH varían de 6.2 a 7.0, una capacidad de intercambio catiónico 30.1 cmol(+)/kg, y el tamaño de partícula predominante es <45 μm (44.2 %), siendo esta fracción donde la mayor cantidad de metales está contenida [As (31.9 %), Cd (26.0 %), Pb (30.7 %), y V (29.1 %)] (SEMARNAT 2004, 2005).

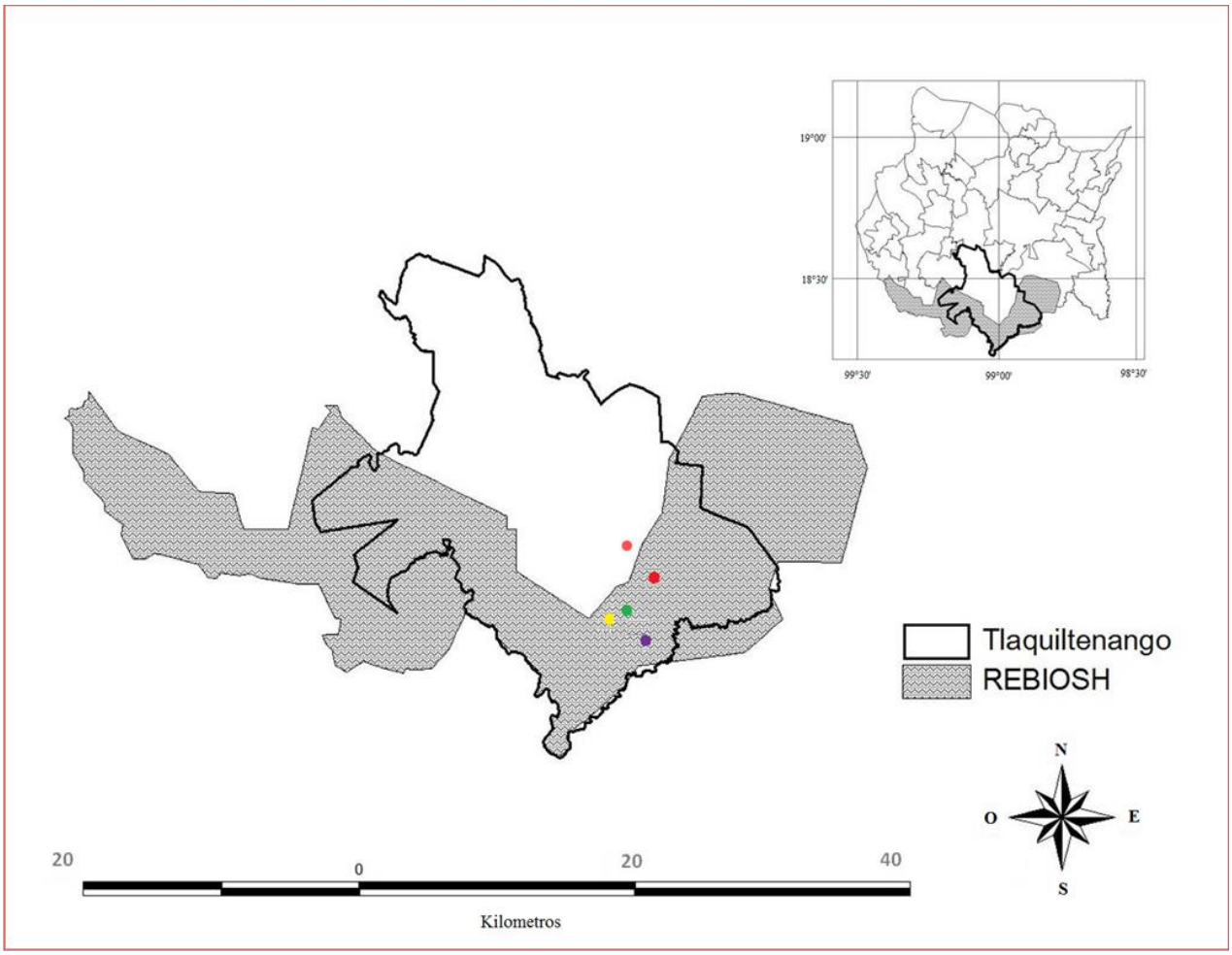

Figura 1. Mapa del estado de Morelos donde se ubica la REBIOSH y los cinco sitios de estudio

Especie de estudio: *Peromyscus melanophrys* (Coues, 1874)

P. melanophrys es de los ratones más grandes del género *Peromyscus*. Difiere de otras especies cercanas a *Peromyscus* de México en que tienen la cola más larga y los lóbulos cerebrales son delgados (Ceballos y Oliva 2005). La coloración del pelaje es muy variada y depende del tipo de sustrato en donde habitan.

Habitan preferentemente en regiones áridas, asociado con yucas, nopal, mezquite, entre otras. Son abundantes en sitios rocosos y se encuentran principalmente en el matorral xerófilo, bosque espinoso y pastizales, en asociaciones vegetales de matorral desértico microfilo y chaparrales, propios de las zonas de contacto entre climas áridos y templados (Ceballos y Oliva 2005).

La reproducción puede ocurrir todo el año pero existen dos picos reproductivos (febrero-marzo y junio-octubre). El tamaño de camada es de 2 a 5 crías, se caracterizan por ser roedores granívoros y se les encuentra desde los 50 hasta los 2,700 m s.n.m. (Hall 1981).

Muestreo de *Peromyscus melanophrys*

Para la colecta de ejemplares de *P. melanophrys*, en cada sitio se realizaron cuatro cuadros de 20 x 100 m. En cada uno se realizó una cuadrícula, donde se colocaron 100 trampas tipo Sherman a ras del suelo separadas entre sí cada cinco metros (en total 400 trampas por sitio). Los muestreos se realizaron a partir del mes de Agosto del 2010, hasta obtener un tamaño de muestra de 20 individuos por sitio (Fig. 2). En todos los sitios, las trampas fueron cebadas con una mezcla de crema de cacahuate, hojuelas de avena y vainilla. Cabe mencionar que las especies más abundantes del género *Peromyscus* dentro de la REBIOSH, son *P. levipes* y *P. melanophrys*. Sin embargo, la primera se encuentra asociada a las zonas montañosas de la REBIOSH, donde el clima es más templado.

Posteriormente, los individuos colectados fueron llevados a la estación biológica de Quilamula, dentro de la REBIOSH, donde se sacrificaron en una cámara letal. A cada animal capturado se le tomaron los siguientes datos: a) lugar y fecha de captura, b) datos morfométricos convencionales (en mm y gr.): longitud total (LT), longitud de la cola (LC), longitud de la pata trasera izquierda (LPTI), longitud de la oreja izquierda (LOI), c) el peso, y d) sexo y etapa reproductiva. Posteriormente, se les extrajeron los órganos (hígado, riñón, testículos u ovarios),

los cuales fueron colocados en bolsas de plástico individuales, etiquetados y transportados en nitrógeno líquido al Laboratorio de Sistemática Molecular del Centro de Investigación en Biodiversidad y Conservación (CIByC), UAEM, Morelos.

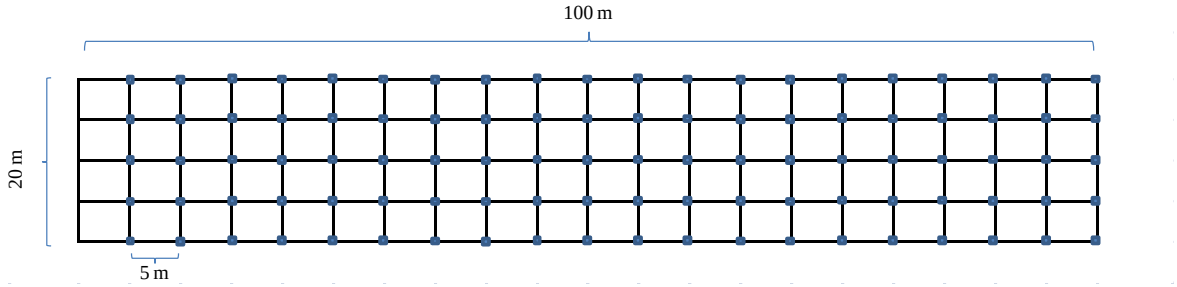

Fig. 2. Diseño del muestreo de *Peromyscus melanophrys* en los sitios de estudio. En cada punto de intersección se colocó una trampa tipo Sherman (100 trampas). En cada lugar de estudio se realizaron 4 cuadriculas (400 trampas totales).

Densidad de *P. melanophrys*

La densidad de los individuos en cada sitio de estudio se estimó de acuerdo con el método de Mares y Ernest (1995), el cual, divide el número de individuos colectados entre el área efectiva de trampeo. Los autores sugieren que para estimar la densidad de roedores, el transecto muestreado debe de incluir un área adicional (10 m). Para este estudio el área efectiva de muestreo fue de 1.76 ha por sitio.

Determinación de metales en riñones de *P. melanophrys*

De los roedores colectados, se tomaron de forma aleatoria cinco individuos asociados a cada sitio de estudio, a estos individuos se les extrajo el riñón, el cual se utilizó para la medición de metales por medio de espectrofotometría de plasma masas (Al, Pb, Cu, As, Cd, Hg, Ni). Se empleó un espectrofotómetro tipo ICP- 820 (MS Series ICP-MS Systems, Bruker, MA, USA).

Todos los análisis se realizaron en el Centro de Investigación de Química Sustentable, Facultad de Química, Universidad Autónoma del Estado de México.

Extracción de ADN y genotipificación de microsatélites

Se extrajo ADN de hígado de cada uno de los individuos colectados mediante el kit de extracción "DNA purification kit" (Promega, USA). Se corroboró la integridad del ADN mediante geles de agarosa al 2%. Se cuantificó la muestra obtenida (biofotómetro, Eppendorf). Ya que la cantidad recomendada para la realización de la técnica de microstatélites es de 30 ng/µl, se hicieron las diluciones correspondientes.

Posteriormente, se probaron 20 secuencias cebadoras de microsatélites (serie PM: 101-112; serie BW: bwc-28, bw-tbx, bw2-110, bw4-1, bw4-5, bw4-7; serie PO: po-35, po-25) (Chiriart et al. 2000; Mullen et al. 2006) vía PCR para detectar polimorfismo.

De las 20 secuencias cebadoras probadas, se seleccionaron únicamente aquéllas que a) amplificaron en todos los individuos, b) presentaron bandas polimórficas entre individuos, y c) presentaron bandas claras y reproducibles, un ejemplo de ello se muestra en la figura 3. En total se seleccionaron ocho secuencias cebadoras de microsatélites, las cuales se marcaron con fluorescencia para posteriormente secuenciar sus productos de PCR en el Laboratorio de Biología Molecular del Instituto de Biología, UNAM (Cuadro 2).

El secuenciador registró el tamaño en pares de bases de cada alelo de microsatélite. Con el tamaño de los fragmentos, se construyó una base de datos la cual fue utilizada para hacer el análisis de los parámetros de diversidad y estructura genética de *P. melanophrys* con los programas ARLEQUIN versión 2000 (Schneider et al. 2000), TFPGA versión 2.0 (Miller 2002) y POPGENE versión 1.31 (Yeh y Rog-cai 1999).

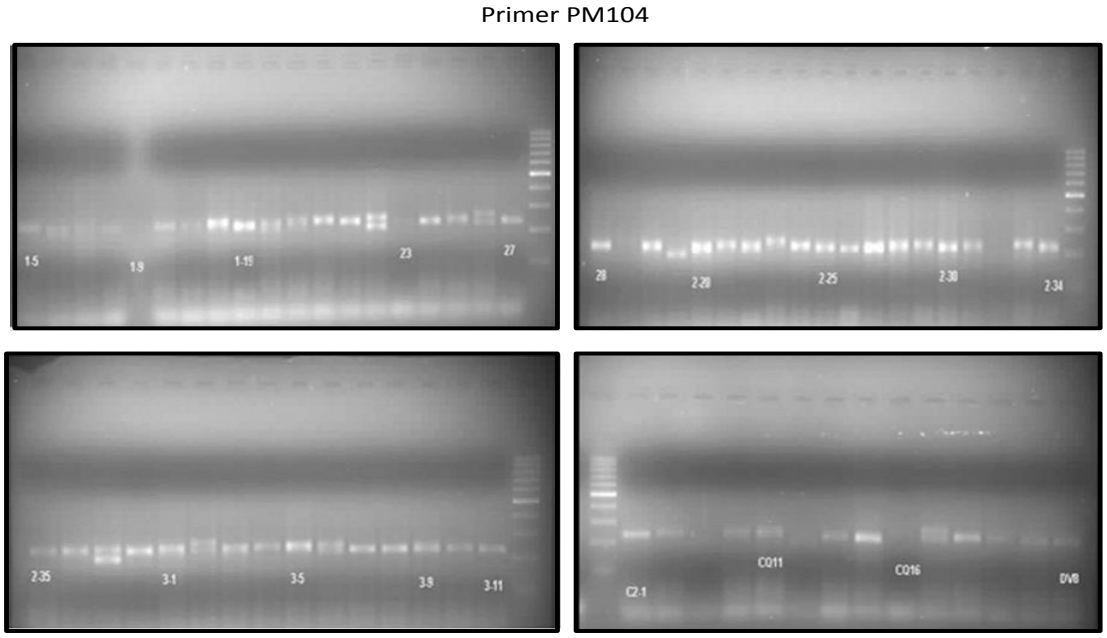

Figura 3. Geles de agarosa al 4% que muestran los productos de PCR amplificados en todos los individuos colectados para la secuencia cebadora (primer) PM104. Las bandas son definidas y se observa el polimorfismo entre individuos. Cada carril denota un individuo distinto.

Cuadro 2.- Lista de secuencias cebadoras seleccionadas y marcadas con fluorescencia para la realización del análisis de la estructura y diversidad genética de *P. melanophrys.* En total se seleccionaron ocho secuencias cebadoras.

Nombre de la Secuencia cebadora	**Secuencia (5'-3')**
PM 103	GCCATTAGTCTATGTGACAG
PM 104	CATAAGGTGGCTCGGAATCA
PM 105	CTGAGCCAAAAGTGGTCCTT
PM 106	CAGGGCTGTAGAGGGAGAAC
PM107	GCCTCTTGTACCCAGTGAAT
PM 109	GAATCCATACACCCATGC
PM 111	ACCCCCGAGTGCTGAGATT
PM 112	GCAGCCTGTATTCTCTCACA

ANÁLISIS DE DATOS

Bioacumulación de metales

Se realizaron análisis de varianza de dos vías (ANOVAs) (Zar 2010) para conocer el efecto de: los sitios de estudio (testigo vs. expuesto), del individuo, de la interacción sitio × individuo sobre la concentración de metales (Al, Pb, Cu, As, y Cd) en los riñones de los individuos de *P. melanophrys*. Además se realizó una prueba de Tukey (Tukey´s honestly significance difference test) para detectar diferencias significativas entre cada promedio de la concentración de metales entre sitios (Zar 2010). Todos los análisis estadísticos se realizaron con el software STATISTICA 6.0 (StatSoft 2000). También se calculó un valor relativo de bioacumulación de metales de los individuos por sitio. Para lo anterior, se dividió la concentración individual del metal *i* (C_i) entre la concentración total registrada ($C_{i\ total}$) y promediándolo con todas las concentraciones registradas de todos los metales, para relacionar la acumulación de metales en los individuos por sitio y la diversidad genética, por lo que, el índice de bioacumulación de metales, definido como "IMBI" por sus siglas en inglés [individual mean (multi-metal) bioaccumulation index] se definió como:

$$\text{IMBI} = \frac{\sum_{i=1}^{n} C_i / C_{iTotal}}{n}$$

Donde n = es el número total de metales, C_i = la concentración individual del metal *i*, $C_i\ total$ = la concentración total del metal *i*. El rango del IMBI va de 0 a 1 (Modificado de Maes et al. 2005). Para comparar la bioacumulación de metales en los individuos de *P. melanophrys* entre sitios de estudio, un análisis de varianza de dos vías ANOVA (Zar 2010) se realizó sobre los valores del IMBI, seguido de un análisis post-hoc de Tukey. Todos los análisis estadísticos se realizaron con el software STATISTICA 6.0 (StatSoft 2000).

Densidad de *P. melanophrys*

Se realizó un análisis de varianza de una vía (ANOVA) para evaluar el efecto del sitio sobre la densidad de *P. melanophrys*. Además, se utilizó un análisis de comparación múltiple (Tukey) para determinar diferencias significativas entre los valores promedio de densidad de individuos entre sitios. Los datos se transformaron logarítmicamente ($X´ = \log X+1$; Zar 2010) para ajustarse a una distribución normal. Los valores de densidad están dados en individuos/hectárea (ind/ha).

Análisis genéticos

Estructura genética: Para conocer como está repartida la variación genética dentro y entre las poblaciones en todos los sitios, se estimó la estructura genética de las poblaciones estudiadas utilizando un análisis molecular de varianza (AMOVA; Excoffier et al. 1992). Los índices de diferenciación genética fueron calculados para ambos modelos mutacionales [paso a paso (R_{ST}) y alelos infinitos (F_{ST})] (Nei 1978).

Distancia genética: Las relaciones genéticas entre las poblaciones de estudio se infirieron con el análisis de dendograma UPGMA (Unweighted pair-group method using arithmetic average) basado en Nei (1978) y el programa POPGENE (Sneath y Sokal 1973; Swofford y Olsen 1990). El árbol se visualizó con el programa TREEVIEW versión 1.52 (Page, 1996). Las pruebas estadísticas de las correlaciones se realizaron con los análisis estadísticos de re-muestreo o "bootstrapping" con un intervalo de confianza del 95% (Weir 1996).

Diversidad genética: se estimó la diversidad genética de *P. melanophrys* empleando las frecuencias alélicas por locus en cada población, utilizando ocho secuencias cebadoras nucleares de SSR. Además, se hizo un promedio de todos los loci para obtener el número promedio de alelos (*A*) y la heterocigosis promedio esperada (*He*). Los datos genéticos fueron analizados con el programa TFPGA versión 1.3 (Miller 2000) y POPGENE (Yeh et al. 1999).

Un análisis de varianza no-paramétrico (Kruskal-Wallis) fue aplicado para determinar si existían diferencias significativas entre los promedios de la heterocigosis esperada (*He*) en las poblaciones estudiadas de *P. melanophrys.* Posteriormente, se realizó un análisis de múltiples comparaciones (Tukey) para determinar las diferencias significativas entre los promedios de las poblaciones analizadas (Zar 2010).

La diversidad genética individual de *P. melanophrys* se cuantificó usando una medida de diversidad genética individual, conocida como "índice de parentesco" o "internal relatedness" (*IR*) (Amos et al. 2001), utilizando ocho loci de SSR. El *IR* está basado en el parámetro propuesto por Queller y Goodnight (1989) exceptuando que para cada locus se comparan dos alelos en lugar de dos pares de alelos. Los valores resultantes se distribuyen de manera normal y están aproximadamente centrados en cero. Los valores negativos indican poca endogamia y menor parentesco entre individuos, mientras que los valores positivos indican mayor endogamia y mayor parentesco entre individuos. Los valores del *IR* se computaron en una plantilla de

EXCEL macro (MicrosoftINC.) escrita en Visual Basic, la cual se encuentra en el sitio web de William Amos (http://www.zoo.cam.ac.uk/zoostaff//meg/amos.htm Departamento de zoología, Universidad de Cambridge, UK). Para este estudio, los valores del *IR* se multiplicaron por (−). Por lo que, los valores debajo del cero denotan individuos con alto parentesco y los valores por arriba del cero denotan individuos con bajo parentesco.

Para conocer la relación entre la diversidad genética (*He, IR*) y la bioacumulación de metales en las poblaciones estudiadas (Al, Pb, Cu, As, Cd e IMBI), se realizaron análisis de regresión. Los valores de *He* y de la concentración de cada metal fueron corregidos como: X = arcoseno (%)1/2 (Zar 2010).

Dado que los parámetros de diversidad genética (*He*, *A*) estuvieron correlacionados significativamente entre ellos (r = 0.090, P = 0.01), los análisis estadísticos solamente se realizaron con la heterocigosis esperada (*He*).

RESULTADOS

Bioacumulación de metales en individuos de *P. melanophrys*

Del total de metales analizados, no se detectaron Hg y Ni en ninguna muestra de tejido examinada. Se registró la misma mezcla de metales (Al, Pb, Cu, As y Cd) en los riñones de *P. melanophrys* en todos los sitios de estudio. En general, los análisis de varianza (ANOVA) detectaron un efecto significativo del sitio (S), del individuo (I) y de la interacción S×I sobre cada concentración de metales y el IMBI en *P. melanophrys.* Los análisis de Tukey mostraron que las concentraciones de metales no difirieron significativamente entre los individuos de ambos sitios testigos. En contraste, la concentración de metales en los riñones de los individuos de los sitios testigo difirió significativamente de todos lugares expuestos (Cuadro 3).

La distribución de la concentración de metales entre sitios fue heterogénea, como se muestra en la figura 4. El rango de los valores obtenidos con el índice de bioacumulación oscilaron entre 0.07 y 0.43 (Fig. 4). Además se encontró un gradiente de bioacumulación para

cada metal y para el IMBI en los individuos de *P. melanophrys* (Cuadro 3), el cual se muestra a continuación:

Testigo 1 = Testigo 2 < Jale 1 < Jale 2 < Jale 3

Estos resultados muestran que en los sitios testigo, los individuos de *P. melanophrys* presentan menor concentración de metales en comparación con los individuos de los sitios expuestos. Los individuos del jale 1 presentaron menor bioacumulación de metales con respecto al jale 2 y los individuos de éste presentaron menor bioacumulación de metales que los individuos del jale 3. Por lo anterior, podemos decir que el jale 3 es el sitio donde se registró la mayor bioacumulación de metales en los individuos de *P. melanophrys.*

Cuadro 3. Concentración promedio de metales (µg/g peso/seco) y desviación estándar en los riñones de *P. melanophrys* en los sitios testigo y expuestos (jales). Se muestran los resultados del análisis de varianza (ANOVA) de una vía para determinar el efecto del sitio de estudio sobre la concentración de metales. N= número de individuos, Al= Aluminio, Pb= Plomo, Cu= Cobre, As= Arsénico, Cd= Cadmio. IMBI = índice de bioacumulación de metales. Letras distintas denotan diferencias significativas con una $P < 0.05$ (Prueba de Tukey).

Sitio	N	Al	Pb	Cu
Testigo 1	5	23.15±3.57 a	0.94±0.17 a	6.82±1.11 a
Testigo 2	5	22.41±5.62 a	0.76±0.25 a	7.07±1.99 a
Jale 1	5	35.61±11.18 b	3.26±0.63 b	11.63±0.42 b
Jale 2	5	42.90±19.64 bc	11.88±5.41 c	16.06±3.20 c
Jale 3	5	46.79±21.51 c	24.33±7.93 d	19.11±5.81 d
ANOVA:	Sitio (S)	$F_{4,100}$ = 59.181 ***	$F_{4,100}$ = 654.073 ***	$F_{4,100}$ = 185.921 ***
	Individuo (I)	$F_{4,100}$ = 16,451 ***	$F_{4,100}$ = 25.187 ***	$F_{4,100}$ = 13.794 ***
	S × I	$F_{16,100}$ = 72.213 ***	$F_{16,100}$ = 26.660 ***	$F_{16,100}$ = 18.097 ***

Sitio	N	As	Cd	IMBI
Testigo 1	5	0.13±0.07 a	0.10±0.07 a	0.066±0.000 a
Testigo 2	5	0.19±0.07 a	0.14±0.11 a	0.069±0.000 a
Jale 1	5	0.46±0.11 b	1.14±0.23 b	0.146±0.003 b
Jale 2	5	0.69±0.18 c	5.43±1.86 c	0.289±0.012 c
Jale 3	5	1.28±0.52 d	7.06±2.15 d	0.430±0.013 d
ANOVA:	Sitio (S)	$F_{4,100}$ = 228.562 ***	$F_{4,100}$ = 1419.047 ***	$F_{4,100}$ = 3010.933 ***
	Individuo (I)	$F_{4,100}$ = 17.534 ***	$F_{4,100}$ = 62.302 ***	$F_{4,100}$ = 38.877 ***
	S × I	$F_{16,100}$ = 34.818 ***	$F_{16,100}$ = 68.383 ***	$F_{16,100}$ = 16.357 ***

*** $P < 0.001$

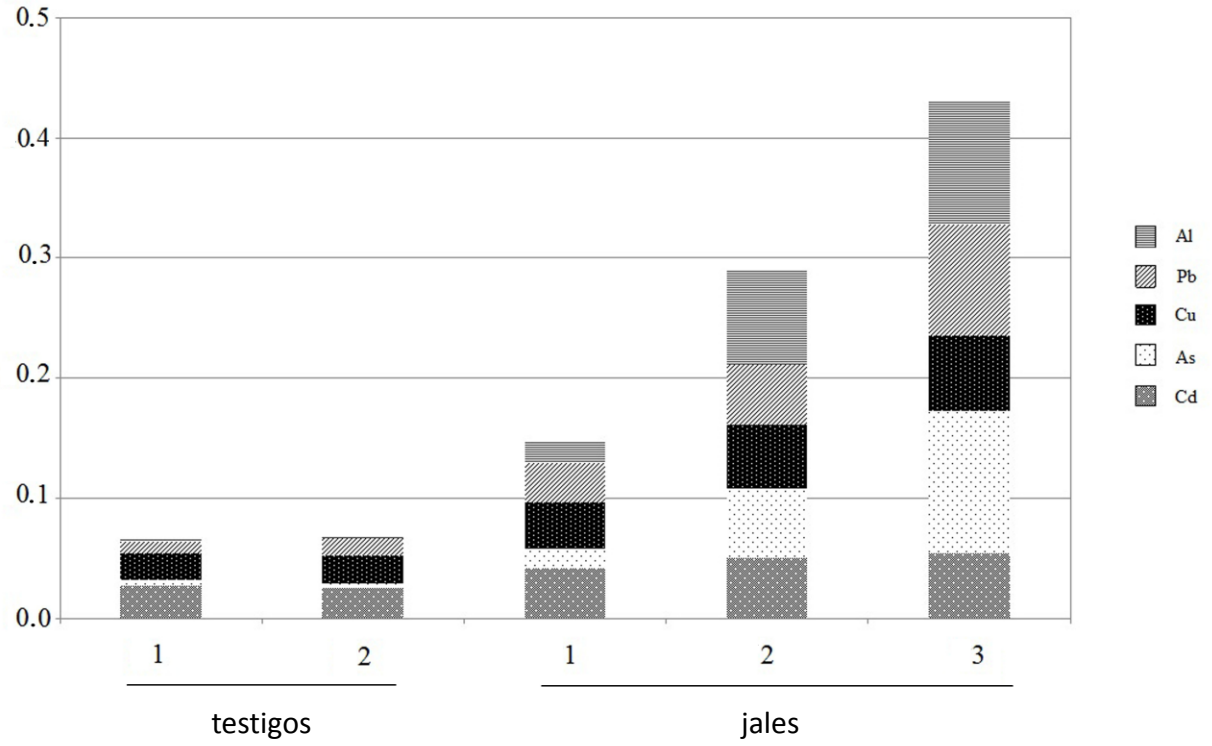

Figura 4. Índice de bioacumulación de metales (IMBI, modificado de Maes et al. 2005) en los riñones de *P. melanophrys* en cada sitio de estudio en Morelos, México. Al= Aluminio, Pb= Plomo, Cu= Cobre, As= Arsénico, Cd= Cadmio.

Densidad de *P. melanophrys*

La densidad promedio de los individuos de *P. melanophrys* fue de 10.35 ind./ha en comparación con 3.70 ind./ha en los sitio expuestos. Se registró un efecto significativo del sitio sobre la densidad de individuos de *P. melanophrys* ($F_{4,40}$ = 10.437, P<0.01). También, la prueba de Tukey (P < 0.05) mostró el siguiente patrón: testigo 2 (11.36 ind./ha) = testigo 1 (9.34 ind./ha) > jale 2 (5.56ind./ha) = jale 1(4.04ind./ha) > jale 3 (1.52ind./ha). Estos resultados indican que la menor densidad de individuos se registró en el jale 3, lo cual fue estadísticamente diferente de los jales 1, 2 y de los sitios testigo.

Diferenciación genética entre poblaciones

Los análisis de varianza molecular (AMOVA) para R_{ST} y F_{ST} mostraron diferencias significativas. En general, para el modelo de mutación paso a paso (SMM), el índice de fijación (R_{ST}) fue estadísticamente diferente para cada sitio (testigo = 0.39, expuesto = 0.33). La mayor variación genética se registró dentro de las poblaciones testigo (81.67 %) y el restante 18.33% entre ellas. Las poblaciones expuestas registraron la mayor variación genética entre ellas (62.84%). Por su parte, el AMOVA para el modelo de mutación por alelos infinitos (IAM), mostró que el índice de fijación (F_{ST}) fue estadísticamente diferente entre sitios testigo y expuestos (testigo = 0.59, expuesto = 0.45). Tanto las poblaciones testigo (9.86%) como las poblaciones expuestas (41.18%) mostraron la mayor variación genética dentro de las poblaciones (Cuadro 4).

El número de migrantes por generación (*Nm*) fue casi seis veces mayor en las poblaciones testigo (5.74) en comparación con las poblaciones expuestas (0.99; Cuadro 4).

Cuadro 4. Estructura y diversidad genética de cinco poblaciones de P. *melanophrys* en Huautla, Morelos, mediante el uso de microsatélites nucleares. N = número de individuos, DS = desviación estándar, *A* = número promedio de alelos, *He* = heterocigosis esperada, *Nm* = número de migrante, R_{ST} y F_{ST} = diferenciación genética. IAM = Modelo de mutación para alelos infinitos. SMM = Modelo de mutación paso a paso.

Población	N	No. loci	*A*	*He*	*Nm*	IAM F_{ST}	SMM R_{ST}
Testigo 1	20	8	11.2	0.786	5.114		
Testigo 2	20	8	10.7	0.837	6.315		
Promedio (± 1DS)			**11.0±0.35**	**0.812±0.04**	**5.714±1.25**	**0.099***	**0.183***
Jale 1	20	8	9.7	0.643	1.205		
Jale 2	20	8	9.2	0.605	1.072		
Jale 3	20	8	8.5	0.421	0.702		
Promedio (± 1DS)			**9.1±0.78**	**0.556±0.12**	**0.993±0.11**	**0.412***	**0.628***

Distancia genética

Los análisis para determinar la distancia genética entre las poblaciones de *P. melanophrys* estudiadas mostraron que ambas poblaciones testigo forman un grupo y son las más similares genéticamente. Ambas poblaciones testigo son más semejantes genéticamente al jale 1. A su vez, la población del jale 1 es más similar al jale 2. La población más disimilar genéticamente entre las 5 poblaciones analizadas fue la del jale 3 (Figura 5)

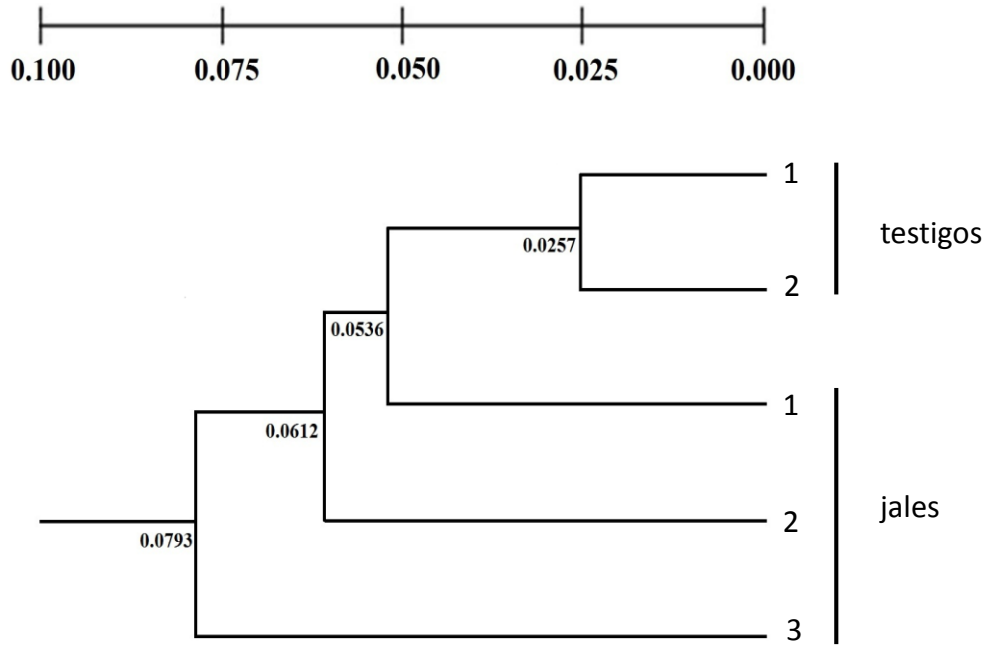

Figura 5. Análisis de similitud genética utilizando el algoritmo de agrupamiento de pares con la media aritmética no ponderada (UPGMA). El dendograma muestra las distancias genéticas entre las cinco poblaciones de *P. melanophrys* estudiadas en Morelos, México. El árbol se calculó a partir de ocho loci nucleares de microsatélites.

Diversidad genética

Todos los loci de microsatélites fueron altamente polimórficos. El número total de alelos por locus varió entre 12 y 17. El promedio de alelos fue de 9.9 y el rango fue de 8.5 a 11.2. El índice de diversidad genética (*He*) mostró el siguiente patrón entre sitios: testigo 2 (*He* = 0.837) > testigo 1 (*He* = 0.786) > jale 1 (*He* = 0.643) > jale 2 (*He* = 0.605) > jale 3 (*He* = 0.421). El análisis de varianza de Kruskal-Wallis reveló diferencias significativas para el índice de diversidad (*He)* entre sitios (H = 19.33, P< 0.001). Además el análisis de comparaciones múltiples de Tukey mostró que este índice fue estadísticamente diferente entre sitios. Los valores

de *He* para todas las poblaciones estudiadas disminuyó conforme aumentó el gradiente de bioacumulación de metales en los individuos de *P. melanophrys* (Cuadro 4).

Relación entre la concentración de metales y la diversidad genética

En general, se detectó una relación negativa y significativa entre el índice de diversidad (*He*) y la concentración de cada metal (Al, Pb, Cu, As, Cd) y el IMBI en los individuos de *P. melanophrys*. El coeficiente de determinación (r^2) varió de 0.78 para Cd a 0.88 para el IMBI (cuadro 5, figura 6). También la relación entre la diversidad genética individual -(*IR*) y la concentración de cada metal (Al, Pb, Cu, As, Cd) y el IMBI en los individuos de *P. melanophrys* mostró un patrón similar. El coeficiente de determinación (r^2) varió de 0.22 para Al a 0.29 para Cd, As y el IMBI (cuadro 5, figura 6). Es decir, conforme aumenta la concentración de cada metal, disminuyen los valores del *IR.*

Cuadro 5. Relación entre la diversidad genética (*He*, *IR*) y la concentración promedio de cada metal y el índice de bioacumulación de metales (IMBI) en las poblaciones de *P. melanophrys* de Huautla, Morelos, México. *He* = heterocigosis esperada, *IR*= diversidad genética individual, r^2 coeficiente de determinación. *F* = Prueba de ANOVA, *P* = nivel de significancia.

	Aluminio			**Plomo**		
	r^2	*F*	*P*	r^2	*F*	*P*
He	0.84	22.911	0.017	0.84	22.911	0.017
IR	0.22	6.428	0.018	0.27	8.579	0.007
	Cobre			**Arsénico**		
He	0.87	27.101	0.014	0.86	25.389	0.015
IR	0.24	7.263	0.013	0.29	9.557	0.005
	Cadmio			**IMBI**		
He	0.78	16.045	0.027	0.88	35.336	0.009
IR	0.29	9.515	0.005	0.29	9.137	0.005

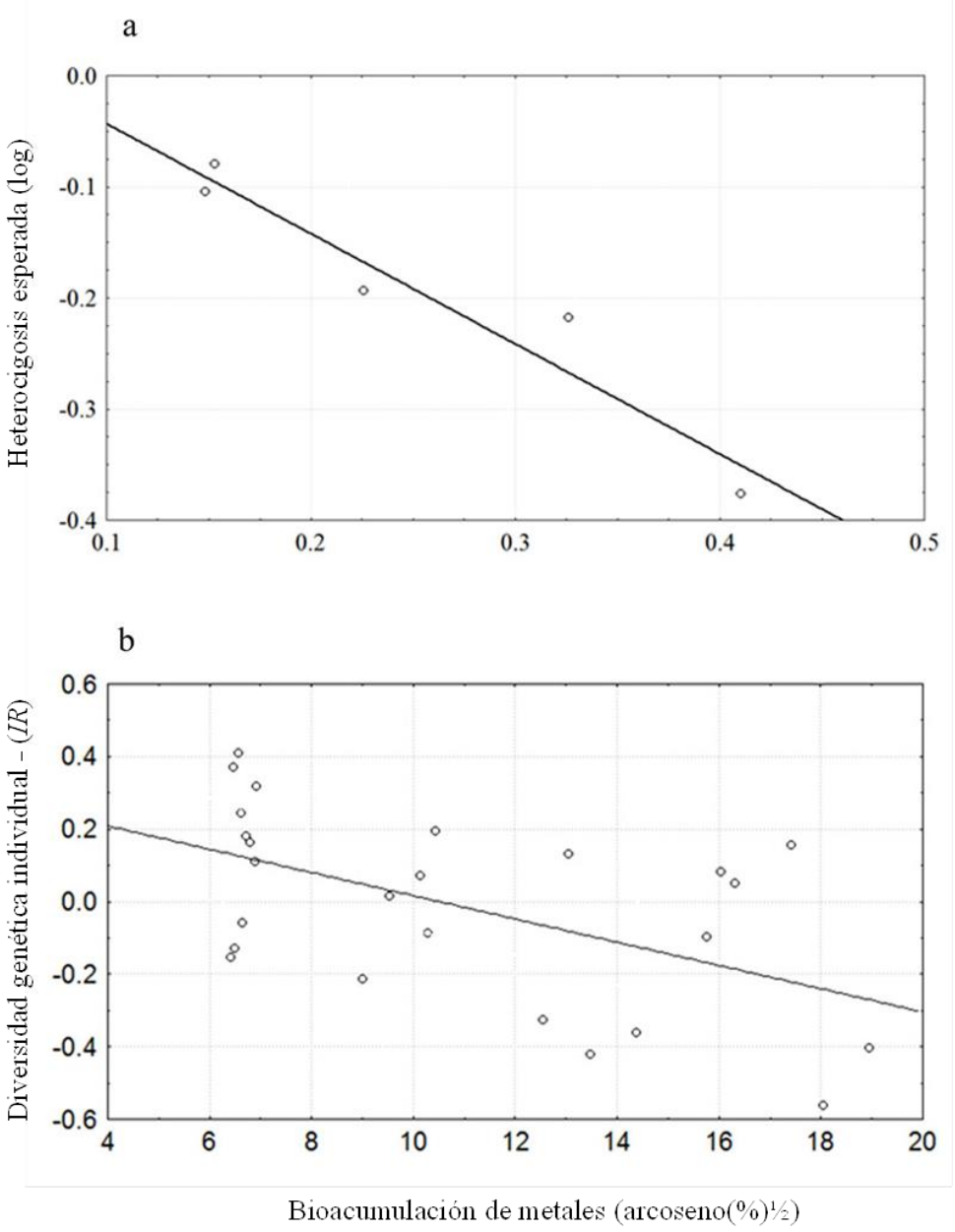

Figura 6a. Relación entre la diversidad genética poblacional de *P. melanophrys* (*He*) y el índice de bioacumulación de metales (IMBI). **b.** Relación entre la diversidad genética individual (*IR*) y el índice de bioacumulación de metales (IMBI) en individuos de *P. melanophrys* en Huautla, Morelos, México.

DISCUSIÓN

La contaminación del ambiente por metales es continua, muy común y tiene consecuencias en todos los niveles de organización biológica, desde la célula hasta el ecosistema (Haimi et al 2006, Mussali et al. 2013). Por lo que, al evaluar exposiciones biológicamente relevantes es de suma importancia buscar biomarcadores en organismos centinela que pueden ayudar a predecir riesgos potenciales, en las poblaciones expuestas y en niveles de organización mayores.

Bioacumulación de metales en individuos de *P. melanophrys*

Los resultados de este estudio demuestran que las poblaciones de *P. melanophrys* que habitan dentro de los jales de Huautla, están expuestas a una mezcla compleja de metales. Además, los individuos que habitan en los sitios testigo también presentaron cantidades detectables de la misma mezcla de metales, lo cual podría deberse a la presencia de metales en los suelos de esta región. Se ha documentado que los suelos de Tlaquiltenango presentan una riqueza natural de minerales azufrados, particularmente plomo y plata y los minerales más comúnmente encontrados son: arsenopirita (FeAsS), galena (PbS), acantita (Ag2S), y calclacita (Cu2S). (Volke et al. 2004, 2005; Secretaría de Economía 2011). Por lo anterior, sugerimos que los individuos de *P. melanophrys* que habitan en ambos sitios testigo están expuestos a metales debido a que se encuentran en los suelos de la región de manera natural.

El gradiente de bioacumulación de metales encontrado en este estudio, revela que de las poblaciones expuestas, la menos contaminada se encuentra en el jale 1 y la más contaminada en el jale 3. Estos resultados se pueden explicar con base en el tamaño y tiempo de abandono de los jales de Huautla; ambos factores son inversamente proporcionales a la concentración de metales en cada jale debido a procesos de lixiviación y erosión. Los individuos del jale más grande y más joven (jale 3) presentaron los niveles más altos de bioacumulación de metales, seguido de los individuos del jale de tamaño intermedio (jale 2) y finalmente por los individuos del jale más pequeño y con más tiempo de abandono. Es decir, los individuos que habitan el jale más grande

y más nuevo (jale 3) tienen mayor concentración de metales ya que este jal se ha lixiviado y erosionado menos en comparación con los jales 1 y 2 que son más viejos y más pequeños y por lo tanto, pudieran presentar menor concentración de metales. También, podemos mencionar que los resultados anteriores muestran que los niveles de bioacumulación de metales registrados entre las poblaciones expuestas refleja acertadamente el nivel de contaminación que existe en el entorno más inmediato o próximo de estas poblaciones. Resultados similares han sido documentados para otras especies de animales no-mamíferos expuestas a metales como *Anguilla anguilla*, (Maes et al. 2005), así como también para especies de mamíferos pequeños como: *Apodemus flavicollis* (Folkeson et al. 1990), *Peromyscus maniculatus*, (Laurinolli y Bendell-Young 1996), *Apodemus sylvaticus* y *clethrionomys glareotus*, (Erry et al. 2000). Sin embargo, hay otros estudios en los que no se pudo demostrar la bioacumulación de metales en mamíferos pequeños que habitan en o cerca de sitios mineros (Pascoe et al. 1994; Vucetich et al. 2001). En general, este estudio demuestra que los individuos de *P. melanophrys* que habitan en los jales de Huautla, Morelos pueden acumular metales del ambiente y los resultados obtenidos apoyan la recomendación de que *P. melanophrys* mostró ser útil como especie centinela para estudios de ecotoxicología.

Densidad de *P. melanophrys*

El presente estudio registró una disminución significativa de la densidad de *P. melanophrys* en el sitio más contaminado (jale 3) en comparación con los sitios testigo. Estos resultados están de acuerdo con lo reportado por Bengtsson et al. (1983) en diferentes especies de lombrices de tierra (*Deschampsia flexuosa, Vaccinium myrtillus* y *Dendrobaena octaedra*) expuestas a metales. Sus datos mostraron que la densidad de las lombrices fué proporcional a la distancia de la fuente de contaminación. Además, *D. octaedra* – la especie más abundante – estuvo ausente en un radio de 1 km de distancia de la fuente de contaminación. Los autores sugieren que la disminución de la densidad de lombrices se debió a una alta toxicidad de metales. Asimismo, se han registrado resultados similares para especies de mamíferos pequeños. Por ejemplo: Dmowski et al. (1995) mostró que la densidad de la población del ratón de campo (*Myodes glareolus*) disminuyó significativamente en sitios contaminados por metales en comparación con lugares no contaminados, sugiriendo el posible efecto de los metales sobre la mortalidad de los individuos que aún no salen del nido. De manera similar, Phelps y McBee (2009) observaron disminución

importante de la densidad poblacional de mamíferos pequeños (*Neotoma floridana* y *Reithrodontomys fulvescens*) que habitaban en sitios mineros. Derivado de los resultados anteriores, se ha evidenciado el efecto de la exposición crónica a metales sobre parámetros demográficos (e.g. densidad poblacional). Algunos autores han demostrado que la contaminación puede influir en la estabilidad de las poblaciones, ya sea incrementando la mortalidad o disminuyendo el éxito reproductivo de los individuos, especialmente cuando se trata de exposición a metales como el cadmio, plomo y arsénico (Peakall 1992; Shore y Douben 1994; Burger 1995; Burger y Gochfeld 1996; Dauwe et al. 2004). Por lo que, alteraciones en la reproducción que deriven en baja fertilidad, baja fecundidad y por lo tanto un bajo éxito reproductivo, pueden afectar la adecuación de la población expuesta. En consecuencia, una menor adecuación de la población puede actuar como una fuerza aceleradora en la reducción del número de sus individuos (Lande 1988; Bickham et al. 2000; Theodorakis 2001; Medina et al. 2007; Phelps y McBee 2009). Más aún, una reducción del tamaño poblacional por debajo del nivel mínimo sustentable, puede llevar a ésta a la extinción por un fenómeno conocido como "derretimiento mutacional" o "mutational meltdown" (Lynch et al. 1995).

Efecto de la bioacumulación de metales sobre la estructura genética de las poblaciones de *P. melanophrys*

En este estudio, los estimadores de estructura genética mostraron que para las poblaciones testigo, solamente el 10% (F_{ST}) y el 18% (R_{ST}) del total de la variación genética, pudo ser atribuida a diferencias entre las poblaciones en una escala microgeográfica (7.5 km). Es decir, la mayor variación genética se atribuye a los individuos dentro de las poblaciones (90% y 82% respectivamente). Estos resultados concuerdan con los valores reportados para otras especies de mamíferos pequeños en escalas geográficas similares, por ejemplo: *Spermophilus mollis* (F_{ST} = 0.10, 8-13 km, Antolin et al. 2001), *Rattus norvegicus* (F_{ST} = 0.07, 0.7-13 km, Gardner-Santana et al. 2009), y *Baiomys musculus* (F_{ST} = 0.11, < 4 km, Vargas 2010). Estos datos sugieren que las poblaciones de pequeños mamíferos se encuentran estructuradas genéticamente en micro escalas geográficas, principalmente por sus limitadas tasas de dispersión (Peakall et al. 2003).

En contraste, los estimadores de estructura genética para las poblaciones expuestas mostraron que el 41 % (F_{ST}) y 63% (R_{ST}) del total de la variación genética, pudo ser atribuida a

diferencias entre las poblaciones. Los datos anteriores coinciden con los valores reportadas para *Perca flavescens* (θ_{ST}=0.36 y R_{ST} =0.39, Bourret et al. 2008) en un gradiente de contaminación por metales (Cu y Cd), utilizando siete loci de microsatélites. Sin embargo, también hay resultados contrastantes, como los reportados por (Berckmoes et al. 2005) para el roedor *Apodemus sylvaticus* quienes, usando 10 loci de SSR registraron que únicamente el 1.58% de la variación genética pudo ser atribuida a diferencias entre las tres poblaciones más contaminadas en comparación con las cuatro poblaciones menos contaminadas, mientras que el 6.02% de la variación genética se observó entre las poblaciones dentro de los grupos en los sitios contaminados (Ag, As, Ad, Cu, y Pb). Por los resultados obtenidos en este trabajo, es posible sugerir que la alta diferenciación genética encontrada en las poblaciones expuestas de *P. melanophrys* podría ser el resultado de alteraciones en los patrones de migración y bajo flujo genético entre ellas (*Nm* = 0.993). En general, nuestros resultados están apoyados por el análisis de agrupamiento, el cual mostró que la población perteneciente al jale 3 fue la más distante genéticamente de ambas poblaciones testigo. Además este mismo análisis indicó que las poblaciones de los sitios contaminados fueron más similares genéticamente entre ellas en comparación con las poblaciones testigo, resultado que está de acuerdo con lo reportado por Yap et al. (2004, 2011). En consecuencia, nosotros proponemos que las medidas de distancia genética entre las poblaciones de *P. melanophrys* pueden servir como un indicador genético potencial para estudiar o monitorear cambios ambientales.

Es importante mencionar que la estructura genética de las poblaciones de *P. melanophrys* en esta región puede ser explicada con base en el patrón de exposición a metales, por lo que el efecto genético a nivel poblacional derivado de estrés ambiental, es uno de los factores principales que afectan la forma en cómo se distribuye la variación genética dentro y entre las poblaciones.

Con respecto al número de migrantes por generación (*Nm*), las poblaciones de *P. melanophrys* de los sitios testigo registraron casi seis (5.7 ± 1.3) migrantes por generación. La tasa de migración está determinada por las características de historia de vida de las poblaciones, como la densidad poblacional. La densidad *de P. melanophrys* en estos sitios fue de 10.35 ind./ha., lo que es similar a otros registros para individuos del género *Peromyscus* dentro de la

REBIOSH (9.18 ind/ha, Cadena 2003). Estas densidades pueden estar reflejando la alta tasa de migración, como es el caso de otras especies de mamíferos pequeños como: *Hypogeonomys antinema* (Sommer 2003) y *Arvicola terrestres* (Berthier et al. 2006).

Por otro lado, el número de migrantes por generación en las poblaciones expuestas fue significativamente menor (0.993) comparado con el registrado en las poblaciones de los sitios testigo. Las bajas tasas de migración entre las poblaciones expuestas a metales puede ser el resultado de una baja densidad poblacional, observación que está de acuerdo con el bajo número de migrantes registrado en las poblaciones expuestas de *P. melanophrys* (promedio 3.70 ind/ha). También, como ya se mencionó anteriormente, los procesos ecológicos que resultan de los efectos genotóxicos de los metales pueden disminuir la adecuación promedio de la población, mediante una reducción del número de descendientes que contribuyen a las siguientes generaciones, lo que resulta en patrones de migración alterados y por lo tanto; menor flujo genético y altas tasas de diferenciación genética (Van Straalen 1999; Van Straalen y Timmermans 2002; Maes et al. 2005). Por todo lo anterior, este estudio demuestra que la contaminación por metales ha alterado (disminuido) los patrones de migración de las poblaciones de *P. melanophrys* que habitan dentro de los jales de Huautla.

En este estudio se obtuvieron los valores de *Nm* a partir de $F_{ST.}$ En general, F_{ST} es un parámetro ideal que refiere la historia de las poblaciones analizadas, el cual arroja información acerca de la importancia evolutiva del flujo y la deriva génica. Es fácil de obtener los datos necesarios para calcular F_{ST} y posteriormente *Nm*. Sin embargo, algunos autores cuestionan este método debido a que se basa en supuestos poco reales, como: a) el tamaño poblacional de todas las poblaciones es el mismo, b) el flujo genético entre poblaciones es simétrico, c) existe un número infinito de poblaciones donde no hay selección ni mutación y d) cada población se encuentra en equilibrio entre migración y deriva (Beerli y Felsenstein 2001; Niegel 2002). Slatkin y Barton (1989) analizaron la relación entre F_{ST} y *Nm,* concluyendo que F_{ST} provee un estimado razonable de *Nm* bajo una variedad de condiciones. Por su parte, Whitlock y McCauley (1999) señalan que hay evidencias de que muchos de los loci a partir de los cuales se estima F_{ST} están sujetos a selección, lo cual podría subestimar o sobreestimar los valores de $F_{ST.}$ Sin embargo, Niegel (2002) retoma lo propuesto por Whitlock y McCauley (1999) y propone que aunque F_{ST} no siempre da estimaciones "precisas", es poco probable que sus valores se

encuentren subestimados o sobreestimados por varios órdenes de magnitud. También argumenta que las poblaciones no necesariamente tienen que ser infinitas ni muy grandes para que F_{ST} de una estimación atinada de *Nm* y que la mutación y selección sólo son importantes cuando las poblaciones son muy grandes. En conclusión, los resultados indican que el uso de F_{ST} para estimar *Nm* es útil, dado que este método tiene la ventaja de incorporar los efectos de todos los componentes históricos de la dispersión y generar un promedio de la variación en la dispersión a través del tiempo (Niegel 2002).

Efecto de la bioacumulación de metales sobre la diversidad genética de las poblaciones de *P. melanophrys*

En el presente trabajo se demostró una correlación altamente significativa entre la bioacumulación de metales y los niveles de diversidad genética registrados en las poblaciones de *P. melanophrys* que residen en los jales de Huautla. El nivel de diversidad genética encontrado para las poblaciones de los sitios testigo (*He* = 0.812) fue comparable con otros valores de *He* estimados en otras poblaciones del género *Peromyscus* (Chirhart et al. 2005, *He* = 0.80 a 0.95 en *P. melanossis;* Mossman y Wasser 2001, *He* = 0.80 en *P. leucopus).* Valores similares se registraron en otras poblaciones de mamíferos pequeños (Van de Zande et al. 2000, *He* = 0.84 en *Microtus oeconomus;* Berthier et al. 2005, *He* = 0.82 en *Arbicola terrestris*, Peakall y Lindenmayer 2006, *He* = 0.86 en *Rattus fuscipes*; Gauffre et al. 2008, *He* = 0.88 en *Microtus arvalis*).

Se registró una relación significativa y negativa entre la diversidad genética (*He, IR*) y los niveles de bioacumulación de metales en *P. melanophrys* (independientemente del tipo de metal o del IMBI). En general, los valores más altos del coeficiente de determinación fueron registrados cuando se analizó la diversidad genética a nivel poblacional (*He*) en comparación con la diversidad genética individual (*IR*). Estos resultados sugieren que los individuos que conforman las poblaciones de *P. melanophrys* presentan un amplio rango de diversidad genética (*IR;* 0.6< 0 >0.6), registrando individuos con valores de *IR* cercanos a cero que nacieron de padres no emparentados, individuos con valores positivos sugiriendo poca endogamia e individuos con valores negativos indicando altos niveles de endogamia.

Adicionalmente, el amplio rango de diversidad genética observada cuando se correlacionaron los valores de *IR* con cada metal o el IMBI, posiblemente es el resultado de diferencias inter-individuales en la bioacumulación de metales (Ma et al. 1991; Smith et al. 2002). Primero, las especies de *P. melanophrys* anidan en el suelo, lo que resulta en un incremento de la exposición a los contaminantes del suelo (Ma et al. 1991). Además, estos roedores se alimentan de semillas que se encuentran principalmente en el suelo, por lo que la ingestión e inhalación incidental de suelos contaminados con distintos contenidos de metales (como es el caso de la distribución heterogénea de metales a lo largo de los jales de Huautla, Fig. 4) puede estar contribuyendo a los efectos observados. Al mismo tiempo, los individuos de *P. melanophrys* que habitan en los jales de Huautla se alimentan de plantas como: *Prosopis laevigata*, *Acacia farneciana* y *Pithecelobium dulcis*, las cuales son hiperacumuladoras de metales como Pb, Cu, y Zn en la región de Huautla, Morelos (datos no publicados). Entonces, para los individuos de *P. melanophrys*, las principales rutas de exposición a metales consisten en el consumo de alimentos y agua contaminada, así como la ingestión e inhalación incidental del suelo. Otros factores asociados con el amplio rango de variación genética individual observada pueden ser: diferencias en el metabolismo de desintoxicación de xenobióticos (Smith 2002), diferencias relacionadas con el género e inter-individuales en el nivel de daño al ADN de estos individuos (Tovar-Sánchez et al. 2012).

La pérdida de diversidad genética observada en las poblaciones expuestas a metales de *P. melanophrys*, es similar a lo reportado para otras especies de animales silvestres expuestas a este tipo de contaminación, por ejemplo: *Littorina brevicula* (Kim et al. 2003), *Pleurocerca canaliculatum* (Benton et al. 2002), *Rana ridibunda* (Matson et al. 2006), *Hattheyella crassa* (Garderström et al. 2008), *Pachygrapsus marmoratus* (Fratini et al. 2008) y *Talitrus saltator* (Ungherese et al. 2010). Estos autores atribuyen la pérdida de diversidad genética en estas especies a selección de ciertos genotipos, es decir, a la selección de genotipos tolerantes y a la eliminación de aquellos intolerantes, a una reducción en el tamaño poblacional y a una disminución del flujo genético entre poblaciones. La pérdida de diversidad genética en poblaciones sometidas a un estrés ambiental se conoce como "erosión genética" y es un factor que se debe tomar en cuenta cuando se estiman los riesgos de las poblaciones expuestas a contaminación ambiental (Van Straalen y Timmermans 2002). En este contexto, Fratini et al.

(2008) encontraron una disminución de la diversidad genética en el cangrejo corredor (*Pachygrapsus marmoratus*) expuesto a una mezcla de metales (As, Pb, Cd, Cu). Resultados similares fueron registrados por Ungherese et al. (2010) en el saltamontes *Talitrus saltator* en contacto con Hg, Cu y Cd. Ambos estudios sugieren que en estas poblaciones ha ocurrido erosión genética. En este sentido, nuestro estudio apoya esta hipótesis al demostrar los efectos negativos de los metales sobre la diversidad genética de las poblaciones expuestas. Sin embargo, otros estudios (Berckmoes et al. 2005) reportan que las poblaciones del ratón de campo (*Apodemus sylvaticus*) pertenecientes a los sitios más contaminados por metales no difieren de las poblaciones que habitan en los sitios menos contaminados en términos de niveles de heterocigosis. Más aún, estos autores no encontraron una correlación entre las medidas de diversidad genética y el nivel de contaminación por metales pesados y argumentan que el estrés inducido por la contaminación no fue lo suficientemente intenso o que el tiempo que ha transcurrido desde que empezó la contaminación no ha sido suficiente (más de 50 años) para inducir cambios en la diversidad genética de las poblaciones. También mencionan que el flujo genético puede enmascarar los efectos negativos de la contaminación por metales en la estructura y diversidad genética de la población. Estas observaciones contrastan claramente con nuestros resultados, donde los efectos de estos factores fueron disminuidos, ya que las poblaciones analizadas en este estudio presentaron niveles muy bajos de flujo genético (Nm = 0.99) y una disminución de la densidad.

En general, la pérdida de diversidad genética en las poblaciones que habitan en jales o sitios mineros en comparación con sitios no contaminados, puede tener diversas explicaciones, ya que los efectos de los metales se observan en todos los niveles de organización biológica: a nivel molecular, los metales alteran la molécula del ADN, produciendo daños genéticos (adúctos, rompimientos de cadena doble y sencilla, alteraciones cromosómicas, bases oxidadas etc.) (Scheirs et al. 2006; Bernard 2008; Pra et al. 2008), lo que causa alteraciones celulares y tisulares, resultando en alteraciones sobre la salud del individuo (Valavandis y Vlachogianni 2010). En este contexto, nuestro estudio previo mostró que individuos de *P. melanophrys* que habitan en los jales de Huautla presentaron significativamente más daño genético (RCS) que los individuos del sitio testigo. Además encontramos un efecto de los metales relacionado con el género, en el cual las hembras registraron mayor daño al ADN que los machos (Tovar-Sánchez

et al. 2012). El daño genético que resulta de una exposición crónica a metales puede alterar las células somáticas o germinales. Si las alteraciones ocurren en las primeras, los efectos sobre la salud del individuo podrían causar menor longevidad, alteración en la proporción de tamaños y sexos entre individuos. En contraste, efectos teratógenos y mutaciones heredables se pueden originar si las alteraciones se dan en las células germinales, lo que causaría bajo éxito reproductivo, menor viabilidad y fertilidad, especialmente si las hembras presentan mayor daño genético, como es el caso de las hembras de *P. melanophrys* en Huautla, Morelos. Ambos escenarios resultarían en una disminución de la diversidad genética de las poblaciones expuestas (Bickham et al. 2000). Como resultado, los efectos relacionados con la exposición a metales se observan a nivel de la población (Bickham y Smolen 1994; Shugart y Theodorakis 1998; Theodorakis 2001; Medina et al. 2007), donde una reducción de la diversidad genética podría ser el resultado directo de la exposición a contaminantes ambientales mediante el proceso de selección genotípica. La cual podría favorecer genotipos tolerantes y eliminar aquellos intolerantes, causando entonces cambios genéticos a nivel de las poblaciones (Lefèbvre y Vernet 1990; Deng et al. 2007; Yap et al. 2007).

Adicionalmente, cambios en los parámetros demográficos como una baja densidad de la población expuesta puede reducir su diversidad genética (Hebert y Murdoch-Luiker 1996; Bickham et al. 2000), como lo reportan Hebert y Murdoch-Luiker (1996), donde el análisis de poblaciones de peces expuestas a contaminación reveló una depresión en la diversidad de haplotípos y alteraciones demográficas en éstas. Estos resultados apoyan nuestros registros acerca de la baja densidad observada en las poblaciones expuestas de *P. melanophrys* en comparación con los sitios no contaminados, donde la población más contaminada registró la densidad más baja así como los menores niveles de diversidad genética.

Otra causa que puede explicar la reducida diversidad genética son las alteraciones en los patrones de migración de las poblaciones expuestas, en las que bajas tasas de migración pueden reducir la diversidad genética (Staton et al. 2001; Van Straalen y Timmermans 2002). En nuestro estudio, las poblaciones de *P. melanophrys* de los sitios testigo mostraron casi seis veces más migrantes por generación en comparación con las poblaciones expuestas. Tomando en cuenta la regla de “un migrante por generación es suficiente para mantener iguales dos sub-poblaciones” y

estimados más recientes que sugieren que "de cinco a diez migrantes por generación son requeridos para homogeneizar la diversidad genética de varias poblaciones animales" (Mills y Allendorf 1996), las poblaciones testigo mostraron un número suficiente de migrantes por generación para homogeneizar los niveles de diversidad genética. Mientras que las poblaciones expuestas mostraron niveles muy bajos de flujo genético para mantener niveles más altos de diversidad genética. Estos resultados apoyan la pérdida de diversidad genética en las poblaciones expuestas debido a una reducción en las tasas de migración, disminuyendo el flujo genético entre poblaciones cercanas, lo que tendería a aumentar el efecto de un cuello de botella (Belfiore y Anderson 1998).

Todos los resultados mencionados anteriormente, podrían explicar el aumento en la endogamia de las poblaciones expuestas (*IR*) en comparación con las poblaciones testigo, donde si hay flujo genético, poca endogamia y baja diferenciación genética. Estas observaciones concuerdan con la propuesta de que la contaminación química puede favorecer un aislamiento reproductivo de las poblaciones expuestas donde altos niveles de endogamia han sido registrados (Nacci y Hoffman 2008; Brown et al. 2009). Derivado de lo anterior, nuestros resultados también soportan la sugerencia de que los jales y sitios mineros abandonados generalmente contienen altas concentraciones de metales y pueden ser considerados como "islas ecológicas" (Deng et al. 2007). Particularmente, la endogamia aumenta en poblaciones silvestres de tamaño pequeño como resultado de un cuello de botella debido a contaminación ambiental (Bickham et al. 2000). Para el caso de pequeños mamíferos (i.e. *Sorex araneus*, *Peromyscus leucopus*; Stockley et al. 1993), la endogamia también puede verse favorecida por sus bajas tasas de dispersión, lo que refuerza el uso de pequeños mamíferos como organismos centinelas para evaluar el efecto genético poblacional de la contaminación ambiental.

En resumen, una población expuesta crónicamente a contaminación por metales, que presenta altos niveles de daño genético, densidad reducida y bajo flujo genético puede experimentar una pérdida de diversidad y altas tasas de diferenciación genética y finalmente derivar en un cuello de botella. Si es así, la diversidad genética de estas poblaciones puede verse reducida en comparación con poblaciones no expuestas como consecuencia de la deriva génica (VanStraalen y Timmermans 2002).

Finalmente, este estudio apoya la hipótesis de la "erosión genética" al demostrar los efectos negativos de la contaminación ambiental sobre la diversidad genética de las poblaciones de *P. melanophrys.* También, este estudio reveló que el efecto de la exposición ambiental a metales es uno de los principales factores que afectan la forma en como la diversidad genética se distribuye dentro y entre las poblaciones de *P. melanophrys.*

CONCLUSIONES

El presente estudio demostró que las poblaciones de *P. melanophrys* que habitan en los jales de Huautla, Morelos, están expuestas de manera crónica a una mezcla compleja de metales, los cuales se bioacumularon en los riñones de estos mismos individuos. Derivado de lo anterior, podemos concluir lo siguiente:

- Se registró un gradiente de bioacumulación de metales en los riñones de *P. melanophrys*, el cual dependió del tamaño del jale al cual pertenecían los individuos analizados.

- Se observó disminución en la densidad de las poblaciones expuestas con respecto a las poblaciones testigo. La población que registró el mayor índice de bioacumulación de metales, fue la que menor densidad registró.

- Se determinó que las poblaciones expuestas presentaron altos niveles de diferenciación genética entre poblaciones con respecto a las poblaciones testigo.

- La población de *P. melanophrys* más contaminada fue la más disimilar genéticamente con respecto a las poblaciones testigo.

- Se registraron alteraciones en los patrones de migración de las poblaciones expuestas con respecto a las poblaciones testigo. La población más contaminada registró los menores valores de flujo genético.

- Las poblaciones expuestas presentaron aumento en los niveles de endogamia con respecto a las poblaciones testigo.

- Se encontró una relación negativa y significativa entre los niveles de diversidad genética y la bioacumulación de metales en las poblaciones de *P. melanophrys.*
- Se demostró que el efecto de la exposición ambiental a metales es uno de los principales factores que afectan la forma en cómo la diversidad genética se distribuye dentro y entre las poblaciones de *P. melanophrys*
- Nuestros resultados apoyan la consideración de que *P. melanophrys* puede ser utilizado como organismo centinela o bioindicador en estudios ecotoxicológicos
- Los microsatélites resultaron ser un marcador útil para revelar cambios genéticos en poblaciones de *P. melanophrys* en escalas microgeográficas
- Este estudio demuestra la utilidad de los parámetros de estructura y diversidad genética como biomarcadores de la salud poblacional

PERSPECTIVAS

El gran reto en estudios de ecotoxicología es demostrar la relación que existe entre los efectos de los contaminantes y las repuestas biológicas en niveles de organización mayores. Para lograr lo anterior, se sugiere lo siguiente:

1) Medir las concentraciones de los contaminantes químicos involucrados en suelo, agua o aire, además de describir la naturaleza química del contaminante ambiental, más que referirse a la contaminación como "mezclas complejas" o "sitios con altos niveles de metales pesados".

2) Complementar los datos ecotoxicológicos de las poblaciones con análisis de la comunidad y el ecosistema -lo cual sería un complemento natural- además de ser importante ya que los biomarcadores en diferentes niveles de organización proveen distintos tipos de información necesaria para un análisis más robusto del riesgo ecológico que representa la contaminación por metales. Por lo que, la utilización de biomarcadores en estudios de ecotoxicología es una prioridad que se debe fortalecer. Particularmente, en estudios futuros se deben emplear biomarcadores de dosis interna (concentración de metales en tejidos, órganos o

fluidos) de efectos tempranos (ensayos de genotoxicidad) y de susceptibilidad (polimorfismos genéticos), en conjunto con biomarcadores a nivel de la población, la comunidad y el ecosistema. La integración de un enfoque de "multi-biomarcadores" en estudios ecotoxicológicos puede proveer evidencias sólidas de los efectos ecológicos de la contaminación sobre la salud del ecosistema.

3) Expandir el uso de organismos centinelas a otras especies animales. Además, el utilizar diferentes organismos centinelas o un enfoque de "multi-especies", facilitaría la extrapolación de los efectos moleculares y ecológicos observados en organismos modelo a un mayor número de especies, además de extrapolar los resultados a niveles de organización mayores.

4) En trabajos de campo, el diseño experimental debe incluir sitios de estudio que presenten gradientes de contaminación ambiental, además de varios sitios testigo, para poder identificar mejor las relaciones causa-efecto.

5) Aumentar el muestreo de poblaciones no contaminadas que se encuentren geográficamente cercanas a las poblaciones expuestas. Este diseño experimental permite que los cambios observados en la estructura y diversidad genética de las poblaciones sean el resultado de la exposición a un ambiente contaminado. El análisis de poblaciones cercanas geográficamente, disminuye la posibilidad de que los efectos observados dentro y entre las poblaciones sean debidos a procesos filogeográficos.

6) Bickham y cols. (2000) sugieren que los cambios genéticos de las poblaciones expuestas son independientes del mecanismo de toxicidad de los contaminantes e indicadores de efectos transgeneracionales, por lo que estos cambios representan el biomarcador de efecto más importante. Los cambios en el reservorio genético de las poblaciones, especialmente la pérdida de diversidad genética, pueden ser permanentes (dependiendo del tamaño poblacional y la tasa de mutación), ya que una vez que se pierde la variabilidad, aunque la población se pudiera recuperar, no podrá hacerlo como era antes del impacto ambiental. Además, como ya se mencionó, cambios en la diversidad genética pueden ser utilizados como un biomarcador de la salud del ecosistema. Por todas las razones anteriores, los estudios de ecotoxicología se deben enfocar en la búsqueda de nuevos biomarcadores, por ejemplo; biomarcadores de efectos permanentes, los cuales nosotros los definimos como: "cambios o alteraciones en procesos

químicos o biológicos que una vez alterados ya no se recuperaran o no son lo que eran originalmente y resultaran en efectos permanentes sobre las poblaciones". En este sentido, las alteraciones en variabilidad genética pudieran utilizarse como un biomarcador de efectos permanentes.

7) Realizar diseños experimentales para que los estudios ecotoxicológicos y los biomarcadores tengan un enfoque holístico. Una posibilidad para lograr lo anterior, es el uso de tecnologías genómicas para incrementar el entendimiento del efecto de los agentes tóxicos en poblaciones naturales. En este sentido, la "ecotoxicogenómica" aplica el uso de estas tecnologías en donde la expresión de cientos o miles de genes (genómica), proteínas (proteómica) y metabolitos (metabolómica) se analizan simultáneamente. Estas metodologías le agregan mayor valor a los diseños experimentales clásicos, dado que proveen información que integra las variables involucradas en las respuestas moleculares ante la exposición. Además se pueden utilizar como "señales tempranas de alerta" para clasificar mejor a los contaminantes ambientales y para entender mejor sus mecanismos de acción, así como para la búsqueda de nuevos biomarcadores. También, estas metodologías permiten extrapolar los resultados de laboratorio a estudios de campo y de algunas especies centinelas al todo el ecosistema.

Finalmente, ya que la diversidad es la base de la adaptación por selección natural y uno de los pilares de la biodiversidad (Anderson et al. 1994; Van Straalen y Timmermans 2002), debemos poner atención a cualquier situación que la amenace, tal como es el caso de la exposición a metales. Puesto que demostrar respuestas bioquímicas, genéticas, o fisiológicas debido a la exposición de contaminantes ambientales no es suficiente para prevenir la extinción o pérdida de diversidad de vida silvestre; se debe emprender un esfuerzo verdadero para revelar los efectos en las poblaciones, comunidades y ecosistemas. Para ello, se requieren investigaciones multidisciplinarias en conjunto con diseños experimentales más robustos y detallados para resolver problemas complejos en ecotoxicología genética.

LITERATURA CONSULTADA

Albert AL. Contaminación ambiental: Origen, clases, fuentes y efectos (2004) En: Toxicología Ambiental. Albert LA (Ed). Universidad Autónoma de Ciudad Juárez, Chihuahua, México.

Amos W, Worthington W, Fullard K, Burger TM, Croxall JP, Bloch D, Coulson T (2001) The influence of parental relatedness on reproductive success. Proc R Soc Lond B 68:2021–2027

Anderson S, Sadinski W, Shugart L, Brussard P, Depledge M, Ford T, Hose J, Stegeman J, Suk W, Wirgin I, Wogan G (1994) Genetic and molecular ecotoxicology: A research framework. Environ Health Perspect 102:3–8

Anthony R, Koslowski R (1982) Heavy metals in tissues of small mammals inhabiting waste-water-irrigated habitats. J Environ Qual 11:20–22

Antolin MF, Van Horne B, Berger MD, Holloway AK, Roach JL, Weeks RD (2001) Effective population size and genetic structure of a Piute ground squirrel (*Spermophilus mollis*) population. Can J Zool 79:26–34

Antón A, Serran T, Angulo E, Ferrero G, Rallo A (2000) The use of two species of crayfish as environmental quality sentinels: The relationship between heavy metal content, cell and tissue biomarkers and physico-chemical characteristics of the environment. Sci Total Environ 249:239-251

Arif I, Khan H (2009) Molecular markers for biodiversity analysis of wildlife animals: A brief review. Animal Biodiv Conserv 32:9–17

Athrey N, Leberg P, Klerks P (2007) Laboratory culturing and selection for increased resistance to cadmium reduce genetic variation in the least killfish, *Heterandria Formosa*. Environ Toxicol Chem 26:1916–1921

Baranski B (1987) Effect of cadmium on prenatal development and on tissue cadmium, copper, and zinc concentrations in rats. Environ Res 42:54–62

Barret SC, Kohn JR (1991) Genetic and evolutionary consequences of small populations in plants: implications for conservation. En: Falk DA, Holsinger K (Eds) Genetics and Conservation of Rare Plants. Oxford University Press. N.Y. pp 3–30

Basta NT, Ryan JA, Chaney RL (2005) Trace element chemistry in residual-treated soil: Key concepts and metal bioavailability. J Environ Qual 34:49–63

Basu N, Scheuhammer AM, Bursian SJ, Elliott J, Rouvinen-Watt K, Chan HM (2007) Mink as a sentinel species in environmental health. Environ Res. 103:130–144

Beeby A (2001) What do sentinels stand for?. Environ Pollut 112:285–298

Beerli P, Felsenstein J (1999) Maximum-likelihood estimation of migration rates and effective population numbers in two populations using a coalescent approach. Genet 152:763–773

Belfiore N, Anderson S (1998) Genetic patterns as a tool for monitoring and assessment of environmental impacts: The example of genetic ecotoxicology. Environ Monit Assess 51:465–479

Belfiore N, Anderson S (2001) Effects of contaminants on genetic patterns in aquatic organisms: A review. Mutat Res 489:97–122

Bengtsson G, Nordström S, Rundgren S (1983) Population density and tissue metal concentration of lumbricids in forest soils near a brass mill. Environ Pollut A 30:87–108

Benton M, Malott M, Trybula J, Dean D, Guttman S (2002) Genetic effects of mercury contamination on aquatic snail populations: Allozyme genotypes and DNA strand breakage. Environ Toxicol Chem 21:584–589

Berckmoes V, Scheirs J, Jordaens K, Blust R, Backeljau T, Verhagen R (2005) Effects of environmental pollution on microsatellite DNA diversity in wood mouse (*Apodemus Sylvaticus*) populations. Environ Toxicol Chem 24:2898–2907

Bernard A (2008) Biomarkers of metal toxicity in population studies: Research potential and interpretation issues. J Toxicol Environ Health Part A 71:1259–1265

Berthier K, Galan M, Foltete JC, Charbonnel N, Cosson JF (2005) Genetic structure of the cyclic fossorial water voles (*Arvicola terrestris*): Landscape and demographic influences. Mol Ecol 14:2861–2871

Berthier K, Charbonnel N, Galan M, Cosson JF (2006) Migration and recovery of the genetic diversity during the increasing density phase in cyclic vole populations. Mol Ecol 15: 2665–2676

Bervoets L, Blust R (2003) Metal concentrations in water, sediment and gudgeon (*Gobio gobio*) from a pollution gradient: relationship with fish condition factor. Environ Pollut 126:9–19

Beyer W, Pattee O, Sileo L, Hoffman D, Mulhern B (1985) Metal contamination in wildlife living near two zinc smelters. Environ Pollut Series A 38:63–86

Beyer W, Storm G (1995) Ecotoxicological damage from zinc smelting at Palmerton, Pennsylvania. En: Hoffman DJ, Rattner BA, Burton GA, Cairns JC (eds) Handbook of ecotoxicology. CRC Press Inc, Boca Raton. pp 596–608

Bickham J, Smolen M (1994) Somatic and heritable effects of environmental genotoxins and the emergence of evolutionary toxicology. Environ Health Persp 102:25–28

Bickham J, Sandhu S, Hebert P, Chikhi L, Athwal R (2000) Effects of chemical contaminants on genetic diversity in natural populations: Implications for biomonitoring and ecotoxicology. Mutat Res 463:33–51

Bickham JW (2011) The four cornerstones of Evolutionary Toxicology. Ecotoxicology 20:497–502. Doi: 10.1007/s10646-011-0636-y

Bisser J, Vogel L, Berger J, Hjelle B, Loew S (2004) Effects of heavy metals on immunocompetence of white-footed mice (*Peromyscus leucopus*). J Wildlife Dis 40:173–184

Bourret V, Couture P, Campbell P, Bernatchez L (2008) Evolutionary ecotoxicology of wild yellow perch (*Percha flavescens*) populations chronically exposed to a polymetallic gradient. Aquat Toxicol 86:76–90

Brooks S, Lyon B, Goodsir F, Bignell J, Thain J (2009) Biomarker responses in mussels, an integrated approach to biological effects measurements. J Toxicol Env Health A 72: 196–208

Brown AR, Hosken DJ, Balloux F, Bickley LK, LePage G, Owen SF, Hetheridge MJ, Tyler CR (2009) Genetic variation, inbreeding and chemical exposure–combined effects in wildlife and critical considerations for ecotoxicology. Phil Trans R Soc B 364:3377–3390

Burger J (1995) Heavy metal and selenium levels in feathers of herring gulls (*Larus argentatus*): differences due to year, gender, and age at Captree, Long Island. Environ Monit Assess 38:37–50

Burger J, Gochfeld M (1996b) Heavy metal and selenium levels in Franklin's gull (*Larus pipixcan):* parents and their eggs. Arch Environ Contam Toxicol 30:487–491

Burger J, Gochfeld M (2004) Marine birds as sentinels of environmental pollution. Ecohealth 1:263–274.

Cadena M (2003) Efecto de la perturbación y estacionalidad en la comunidad de roedores en una salva baja caducifolia en Morelos, México. Tesis de Maestría. Universidad de las Américas de Puebla.

Carleton MD, Musser GG (2005) Order Rodentia. En: Wilson DE, Reeder DM (eds), Mammal species of the world a taxonomic and geographic reference. John Hopkins University Press. Baltimore. pp. 894–1531

Ceballos G, Oliva G (2005) Los mamíferos de México. Fondo de Cultura Económica. México

Chirhart S, Honeycutt R, Greenbaum I (2000) Microsatellite markers for the deer mouse *Peromyscus maniculatus*. Mol Ecol 9:1661–1686

Clements W (2000) Integrating effects of contaminants across levels of biological organization: An overview. J Aquat Ecosyst Stress Recovery 7:113–116

Cooke J, Andrews S, Johnson M (1990) Lead, zinc, cadmium, and fluoride in small mammals from contaminated grassland established on fluorspar tailings. Water Air Soil Pollut 51:43–54

Coutellec M, Barata C (2011) An introduction to evolutionary processes in ecotoxicology. Ecotoxicology 20:513–23

Dauwe T, Janssens E, Kempenaers B, Eens M (2004) The effect of heavy metal exposure on egg size, eggshell thickness and the number of spermatozoa in blue tit *Parus caeruleus* eggs. Environ Pollut 129:125–129

De Wolf H, Blust R, Backeljau T (2004) The population genetic structure of *Littorina littorea* (Mollusca: Gastropoda) along a pollution gradient in the Scheldt estuary (The Netherlands) using RAPD analysis. Sci Total Environ 325:59–69

Deng J, Liao B, Ye M, Deng D, Lan C, Shu W (2007) The effects of heavy metal pollution on genetic diversity in zinc/cadmium hyperaccumulator *Sedum alfredii* populations. Plant Soil 297:83–92

Diez Lázaro J, Kidd P, Monterroso C (2002) Biodisponibilidad de metales en suelos y acumulación en plantas en el área de Trás-Os-Montes (Portugal): influencia del material original. Edafología 9:313–328

Dmowski K, Kozakiewicz M, Kozakiewicz A (1995) Ecological effects of heavy metal pollution (Pb, Cd, Zn) on small mammal populations and communities. B Pol Acad Biol-Sci 43:1–10

Dobzhansky T, Ayala FJ, Stebbins GL, Valentine JW (1993) Evolución. Ediciones Omega S.A. Barcelona.

Dorado O, Maldonado B, Arias D, Sorani V, Ramírez R, Leyva E (2005) Programa de conservación y manejo reserva de la biosfera Sierra de Huautla. Comisión Nacional de Áreas Naturales Protegidas, México

D´Surney S, Shugart L, Theodorakis C (2001) Genetic markers and genotyping methodologies: An overview. Ecotoxicology 10:201–204

Duan Y, Guttman S, Oris J, Bailer A (2001) Differential survivorship among allozyme genotypes *of Hyalella azteca* exposed to cadmium, zinc or low pH. Aquat Toxicol 54:15–28

Durrant ChJ, Stevens JR, Hogstrand C, Bury NR (2011) The effect of metal pollution on the population genetic structure of brown trout (*Salmo trutta* L.) residing in the River Hayle, Cornwall, UK. Environ Pollut 159:3595–3603

Eeva T, Belskii E, Kuranov B (2006) Environmental pollution affects genetic diversity in wild bird populations. Mutat Res 608:8–15

Eisler R (1997) Zinc hazards to plants and animals with emphasis on fishery and wildlife resources. En: Chemisinoff PN (ed) Ecological issues and environmental impact assessment. Gulf Publ Co, Houston. pp 443–537

Ekschmitt K, Korthals G (2006) Nematodes as sentinels of heavy metals and organic toxicants in the soil. J Nematol 38:13–19

EPA. Environmental Protection Agency (2000). Innovative remediation technologies: Field scale demonstration projects in North America

Erry BV, Macnair MR, Meharg AA, Shore RF (2000) Arsenic contamination in wood mice (*Apodemus sylvaticus*) and bank voles (*Clethrionomys glareolus*) on abandoned mine sites in southwest Britain. Environ Pollut 110:179–187

Excoffier L, Smouse PE, Quattro JM (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131:479–491

Fenech M, Holland N, Chang W, Zeiger E, Bonassi S (1999) The human micro-nucleus project-an international collaborative study on the use of the micronucleus technique for measuring DNA damage in humans. Mutat Res. 428:271–283

Folkeson L, Nyholm HEI, Tyler G (1990) Influence of acidity and other soil properties on metal concentrations in forest plants and animals. Sci Total Environ 96:211–233

Fontdevila A, Moya A (1999) Introducción a la genética de poblaciones. Editorial síntesis S.A. Madrid.

Fox GA, (2001) Wildlife as sentinels of human health effects in the Great Lakes–St. Lawrence basin. Environ Health Perspect. 109: 853–861

Fratini S, Zane L, Ragionieri L, Vannini M, Cannicc S (2008) Relationship between heavy metal accumulation and genetic variability decrease in the intertidial crab *Pachygrapsus marmoratus* (Decapoda; Grapsidae). Estuar Coast Shelf S 79:679–686

Frenzilli G, Nigroa M, Lyons B (2009) The comet assay for the evaluation of genotoxic impact in aquatic environments. Celebrating the 20th Anniversary of the invention of the comet assay. Mutat Res Rev. 681:80–92

Gardeström J, Dahl U, Kotsalainen O, Maxson A, Elfwing T, Grahn M, Bengtsson B, Breitholtz M (2008) Evidence of population genetic effects of long-term exposure to contaminated sediments: A multi-endpoint study with copepods. Aquat Toxicol 86:426–436

Gardner-Santana LC, Norris DE, Fornadel CM, Hinson ER, Klein SL, Glass GE (2009) Comensal ecology, urban landscapes and their influence on the genetic characteristics of city-dwelling Norway rats (*Rattus norvegicus*). Mol Ecol 18:2766–2778

Gauffre B, Estoup A, Bretagnolle V, Cosson F (2008) Spatial correlation structure of a small rodent in heterogeneous landscape. Mol Ecol 17:4619–4629

Gileva EA, Rakitin SB, Fokin MV, Abramson NI, Mukhacheva SV (2008) Microsatellite DNA Variation in Ural Bank Vole Populations. Russ J Ecol. 39:544–547

Gillespie R, Guttman SI (1989) Effects of contaminants on the frequencies of allozymes in populations of the central stoneroller. Environ Toxicol Chem 8:309–317

González-Flores E, Tornero-Campante MA, Sandoval-Castro E, Pérez-Magaña A, Gordillo-Martínez AJ (2011) Biodisponibilidad y fraccionamiento de metales pesados en suelos

agrícolas enmendados con biosólidos de origen municipal. Rev Int Contam Ambie 27:291–301

Guerrero DL (2004) Conceptos básicos en ecología y su relación con la toxicología ambiental. En: Toxicología Ambiental. Lilia LA (Ed). Universidad Autónoma de Ciudad Juárez, Chihuahua, México. pp. 45–60

Gutiérrez-Galindo E, Villaescusa J, Arreola A (1999) Bioacumulación de metales en mejillones de cuatro sitio selectos de la región costera de Baja California. Cienc Mar 25:557–578

Gutiérrez-Ruiz M, Romero F, González-Hernández G (2007) Suelos y sedimentos afectados por la dispersión de jales inactivos de sulfuros metálicos en la zona minera de Santa Bárbara, Chihuahua, México. Rev Mex Cienc Geol 24:170–184

Guttman S (1994) Population genetic structure and ecotoxicology. Environ Health Perspect 102:97–100

Haimi J, Knott K, Selonen S, Laurikainen M (2006) Has long-term metal exposure induced changes in life history traits and genetic diversity of the enchtraeid worm *Cognettia sphagnetorum?*. Environ Poll 140:463–470

Hall ER (1981) The mammals of North America. John Wiley and Sons. New York

Hancock J (1999) Microsatellites and other simple sequences. Genomic context and mutational mechanisms. En: Microsatellites, evolution and applications. Goldstein D, Schlotterer CH (Eds). Oxford University Press. Reino Unido. Pp.3–9

Harper-Arabie R, Wirth E, Fulton M, Scott G, Ross P (2004) Protective effects of allozyme genotype during chemical exposure in the grass shrimp, *Palaemonetes pugio*. Aquat Toxicol 70:41–54

Hartwig A (2000) Recent advances in metal carcinogenicity. Pure Appl Chem. 72:1007–1014

Hartwig A, Asmuss M, Ehleben I, Herzer U, Kostelac D, Pelzer A (2002) Interference by toxic metal ions with DNA repair processes. Env Health Perspect. 110:797–799

Hebert PM, Murdoch-Luiker M (1996) Genetic effects of contaminant exposure-towards an assessment of impacts on animal populations. Sci Total Environ 191:23–58

Hernández S, González A, Sánchez P, Casas A, Oyama K (2006) Estructura y diferenciación genética de poblaciones silvestres y domesticadas de chile del Noroeste de México analizada con isoenzimas y RAPDs. Rev Fitotec Mex. 29:25–29

Hoffmann A, Willi Y (2008) Detecting genetic responses to environmental change. Nat Rev Genet 9:421–429

Husby M, Hausbeck J, McBee K (1999) Chromosomal aberrancy in white-footed mice (*Peromyscus leucopus*) collected on abandoned coal strip mines, Oklahoma, USA. Environ Toxicol Chem 18:919–925

INEGI. Instituto Nacional de Estadística y Geografía (2004) Información Geográfica del Estado de Morelos, México

INEGI. Instituto Nacional de Estadística y Geografía (2009). Información Geográfica del Estado de Morelos, México

INEGI. Instituto Nacional de Estadística Geografía e Informática (2011): Industria minero-metalúrgica en México Boletín de prensa núm. 482/11

INEGI. Instituto Nacional de Estadística Geografía e Informática (2011): La minería en México. Serie Estadísticas Sectoriales.

Jarne P, Lagoda JL (1996) Microstellites, from molecules to populations and back. Tree 11:424–429

Jhonson M, Roberts R, Hutton M (1978) Distribution of lead, zinc, and cadmium in small mammals from polluted environments. Oikos 30:153–9

Jiang ZF, Huang SZ, Han YL, Zhao JZ, Fu JJ (2011) Physiological response of Cu and Cu mine tailing remediation of *Paulownia fortunei* (Seem) Hemsl. Ecotoxicology. doi: 10.1007/s10646-011-0836-5

Jordaens K, De Wolf H, Van Houtte N, Vandecasteele B, Backeljau T (2006) Genetic variation in two land snails *Cepaea nemoralis* and *Succinea putris* (Gastropoda, Pulmonata), from sites differing in heavy metal content. Genetica 128:227–239

Kim S, Rodriguez M, Suh J, Song J (2003) Emergent effects of heavy metal pollution at a population level: *Littorina brevicula* a study case. Mar Pollut Bull 46:74–80

Kim J, Kim K, Ahn J, Ko I, Lee CH (2005) Investigation and risk assessment modeling of As and other heavy metals contamination around five abandoned metal mines in Korea. Environ Geochem Health 27:193–203

Kisseberth W, Sunderberg J, Nyboer R, Reynolds J, Kasten S, Beasley V (1984) Industrial lead contamination of an Illinois wildlife refuge and indigenous small mammals. J Am Vet Med Assoc 185:1309–1313

Kimura M, Crow JF (1964) The number of alleles that can be maintained in a finite population. Genetics 49:725–738

Klaper R, Rees CH, Drevnick P, Weber D, Sandheinrich M, Carvan M (2006) Gene expression changes related to endocrine function and decline in reproduction in fathead minnow (*Pimephales promelas*) after dietary methylmercury exposure. Environ Health Perspect 114:1337–1343

Lagadic L, Caquet T, Ramade F (1994) The role of biomarkers in environmental assessment: Invertebrate populations and communities. Ecotoxicology 3:193–208

Lande R (1988) Genetics and demography in biological conservation. Science 241:1988 455–1460

Lares ML, Orians KS (1997) Natural Cd and Pb variations in *Mytilus californianus* during the up-welling season. Sci Total Environ 197:177–195

Laurinolli M, Bendell-Young L (2006) Copper, zinc, and cadmium concentrations in *Peromyscus maniculatus* sampled near an abandoned copper mine. Environ Contam Toxicol 30:481–486

Layne JN (1968) Ontogeny. In: King JA (ed) Biology of *Peromyscus* (Rodentia). The American Society of Mammalogists, Publication number 2, Provo, Utah, Pp 148–253

Lefèbvre C, Vernet P (1990) Microevolutionary processes on contaminated deposits. En: Shaw J (ed) Heavy metal tolerance in plants: evolutionary aspects. CRC Press. Boca Raton. Pp 285–300

Leonard S, Bower J, Shi X (2004) Metal-induced toxicity, carcinogenesis, mechanisms and cellular responses. Mol Cell Biochem 255:3–10

Levengood J, Heske E (2008) Heavy metal exposure, reproductive activity, and demographic patterns in white-footed mice (*Peromyscus leucopus*) inhabiting a contaminated wetland. Sci Total Environ 389:320–328

Link S, Kensler J, Groopman J (1995) Biomarkers and mechanistic approaches in environmental epidemiology. Ann Rev Publ Health 16:83–103

Loveless M, Hamrick J (1984) Ecological determinants of genetic structure in plant population. Annu Rev Ecol Syst. 15:65–95

Lynch M, Conery J, Burger R (1995) Mutation accumulation and the extinction of small populations. Am Nat 146:489–518

Ma W, Denneman W, Faber J (1991) Hazardous exposure of ground-living small mammals to cadmium and lead in contaminated terrestrial ecosystems. Arch Environ Contam Toxicol 20:266–270

Ma X, Cowles D, Carter R (2000) Effect of pollution on genetic diversity in the Bay Mussel Mytilus galloprovincialis and the acorn bernacle *Balanus gladula*. Mar Environ Res 50:559–563

Maes GE, Raeymaekers JAM, Pampoulie C, Seynaeve A, Goemans G, Belpaire C, Volckaert FAM (2005) The catadromous European eel *Anguilla anguilla* (L.) as a model for freshwater evolutionary ecotoxicology: Relationship between heavy metal bioaccumulation, condition and genetic variability. Aquat Toxicol 73:99–114

Mares MA, Ernest KA (1995) Population and community ecology of small mammals in a gallery forest of central Brazil. Journal of Mammalogy 76:750–768

Marıin-Guirao L, Marín Atucha E, Lloret Barba J, Martínez López J, García Fernández A (2005) Effects of mining wastes on a seagrass ecosystem: Metal accumulation and bioavailability seagrass dynamics and associated community structure. Marine Environ Res 60:317–337

Martínez-Pacheco M. 2008. Evaluación de los efectos genotóxicos de metales presentes en el agua de bebida en la población de Huautla, Morelos. Tesis de licenciatura. Universidad Nacional Autónoma de México. 77p.

Matson C, Lambert M, McDonald T, Autenrieth R, Donnelly K, Islamzadeh A, Politov D, Bickham J (2006) Evolutionary toxicology: Population-level effects of chronic contaminant exposure on the marsh frogs (*Rana ridibunda*) of Azerbaijan. Environ Health Persp 114:547–552

Medina M, Correa J, Barata C (2007) Micro-evolution due to pollution: Possible consequences for ecosystem responses to toxic stress. Chemosphere 67:2105–2114

Mejía J, Carrizales L, Rodríguez V, Jiménez-Capdeville M, Díaz-Barriga F (1999) Un método para la evaluación de riesgos para la salud en zonas mineras. Salud Públ Méx 4:132–140

Miller MP (2000) Tools for populations genetic analyses (TFPGA) 1.3: A window program for the analyses of allozyme and molecular population genetic data computer software distributed by author.

Mills SL, Allendorf FW (1996) The one-migrant-per-generation rule in conservation and management. Coserv Biol 10:150 –1518

Moncur MC, Ptacek CJ, Blowes DW, Jambor JL (2004) Release, transport and attenuation of metals from an old tailings impoundment. Appl Geochem 20:639–659

Morales EO, Carrillo FC (2010) Plan municipal de desarrollo de Tlaquiltenango, Morelos. H. Ayuntamiento de Tlaquiltenango, Morelos. http://www.tlaquiltenango.gob.mx/Transparencia/Obligaciones_Contable_Administrativas/OCA15_Plan_desarrollo_estatal%20doc.pdf. 19 Septiembre 2012

Morgan AJ, Kille P, Sturzenbaum SR (2007) Microevolution and ecotoxicology of metals in invertebrates. Environ Sci Technol 41:1085–1096

Moriarty F (1999) Ecotoxicology: The study of pollutants in ecosystems. Academic Press. Reino Unido

Mossman CA, Waser PM (2001) Effects of habitat fragmentation on population genetic structure in the white-footed mouse (*Peromyscus leucopus*) Can J Zool. 79:285–295

Mullen L, Hirschmann R, Prince K, Glenn T, Dewey M, Hoekstra H (2006) Sixty polymorphic microsatellite markers for the old field mouse developed in *Peromyscus polionotus* and *Peromyscus maniculatus*. Mol Ecol Notes 6:36 –40

Mussali-Galante P, Tovar-Sánchez E, Fortoul T (2007) Cell cycle, P53 and metals. En: Metals and Toxicological Implications in Health. Fortoul T (Ed) Research Signpost. Kerala, India. Pp. 9-13

Mussali-Galante P (2008) Estudio sobre la inducción de daño al ADN en sangre periférica de individuos expuestos a metales en al agua de bebida, en la población de Huautla, Morelos. Tesis de Maestría, Universidad Nacional Autónoma de México

Mussali-Galante P, Tovar-Sánchez E, Valverde M, Rojas del Castillo E (2013) Biomarkers of exposure for assessing environmental metal pollution: from molecules to ecosystems. Rev Int Contam Ambie 29:117–140

Nacci D, Hoffman GR (2008) Genetic variation in population-level ecological risk assessment. In: Barnthouse LW, Munns WR, Sorensen MT Jr. (eds) Population-level ecological risk assessment. Taylor and Francis, New York, Pp 93–112

Nedjoud G, Houria B, Rachid R, Amira D, Reda D (2009) Impact of pollution by industrial metallic dust on bio-accumulator organism *Helix aspersa*. Global Veterinaria 3:276–80

Nei M (1978) Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 89:583–590

Neigel J (2002) Is F_{ST} obsolete? Conserv Genet 3:167–173

NOM-127-SSA1-1994. Límites máximos permisibles de metales en suelos residenciales e industriales. Secretería De Salud. México.

NRC. National Research Council (1991) Animals as Sentinels of Environmental Health Hazards. Committee on Animalas Monitors of Environmental Hazards. National Academy Press, Washington, DC.

Ohta T, Kimura M (1973) A model of mutation appropriate to estimate the number of electrophoretically detectable alleles in a finite population. Genet Res 22: 201–204

Page DM, (1996) TreeView: an application to display phylogenetic trees on personal computers. Computer Applications in the Biosciences, 12: 357–358.

Pascoe GA, Blanchet RJ, Linder G, Lande R (1988) Bioavailability of metals and arsenic to small mammals at a mining waste-contaminated wetland. Genetics and demography in biological conservation. Science 241: 455–1460

Peakall, D.B. (1992) Animal Biomarkers as Pollution Indicators. Ecotoxicological Series No.1. Chapman y Hall, Reino Unido.

Peakall D (1994) The role of biomarkers in environmental assessment. Ecotoxicology 3:157–160

Peakall R, Ruibal M, Lindmeyer D (2003) Spatial correlation analysis offers new insights into gene flow in the Australian bush rat, *Rattus fuscipes*. Evolution 57:118–295

Peakall R. Lindmeyer D (2006) Genetic insights into population recovery following experimental perturbation in a fragmented landscape. Biol Conserv 132:520–532

Peles JD, Towler WI, Guttman SI (2003) Population genetic structure of earthworms (*Lumbricus rubelluz*) in soils contaminated by heavy metals. Ecotoxicology 12:379–386

Phelps, K.L. and K. McBee. (2009) Ecological Characteristics of Small Mammal Communities at a Superfund Site. American Midland Naturalist 161:57-68

Piñero D, Barahona A, Eguiarte L, Rocha A, Salas R (2008) La variabilidad genética de las especies: aspectos conceptuales y sus aplicaciones y perspectivas en México. En: Capital natural de México, Conocimiento actual de la biodiversidad. CONABIO, México. Pp 415–435

Pra D, Rech-Frenke SI, Giulian R, Yoneama ML, Ferraz-Diaz J, Erdtmann B, Pegas-Henriques JA (2008) Genotoxicity and mutagenicity of iron and copper in mice. Biometals 21:289–297

Qian Y, Castranova V, Shi X (2003) New perspectives in arsenic-induced cell signal transduction. J Inorganic Biochem. 96:271–278

Queller DC, Goodnight KF (1989) Estimating relatedness using genetic markers. Evolution 43:258–275

Ramos-Arroyo Y, Prol-Ledesma R, Siebe-Grabach CH (2004) Características geológicas y mineralógicas e historia de extracción del Distrito de Guanajuato, México. Posibles escenarios geoquímicos para los residuos mineros. Rev Mex Cien Geol 21:268–28

Ramos-Arroyo Y, Siebe-Grabach CH. 2006. Estrategia para identificar jales con potencial de riesgo ambiental en un distrito minero: Estudio de caso en el distrito minero de Guanajuato, México. Rev Mex Cien Geol 23 (1): 54-74

Reyna-Rosas E. 2009. Evaluación de los cambios nucleares en el epitelio bucal de los individuos expuestos a metales en el agua de bebida en Huautla, Morelos. Tesis de licenciatura. Universidad Nacional Autónoma de México. 73p.

Ricketts HJ, Morgan AJ, Spurgeon DJ, Kille P (2004) Measurement of annetocin gene expression: a new reproductive biomarker in earthworm ecotoxicology. Ecotox Environ Safety 57:4–10

Roark S, Andrews J, Guttman S (2001) Population genetic structure of the western mosquitofish *Gambusia Affinis*, in a highly channelized portion of the San Antonio River in San Antonio, TX. Ecotoxicology 10:223–227

Rogstad S, Keane B, Collier M (2003) Minisatellite DNA mutation rate in dandelions increases with leaf-tissue concentrations of Cr, Fe, Mn, and Ni. Environ Toxicol Chem 22:2093–2099

Rojas E (2009) Special issue on the 20th anniversary of the comet assay. Mutat Res Rev. 681:1–2

Romero FM, Armienta MA, González-Hernández G (2007) The solid-phase control on the mobility of potentially toxic elements in an abandoned lead/zinc mine tailings impoundment, Taxco, Mexico. Appl Geochem 22:109–127

Romero FM, Armienta MA, Gutiérrez ME, Villaseñor G (2008) Factores geológicos y climáticos que determinan la peligrosidad y el impacto ambiental de jales mineros. Rev Int Contam Ambient 24:43–54

Romero FM, Gutiérrez-Ruiz M (2010) Estudio comparativo de la peligrosidad de los jales en zonas mineras localizadas en el sur y centro de México. Bol Soc Geol Mex. 62:43–53

Ross K, Cooper N, Bidwell J, Elder J (2002) Genetic diversity and metal tolerance of two marine species: A comparison between populations from contaminated and reference sites. Mar Poll Bull 44:671–679.

Rossman T (2003) Mechanism of arsenic carcinogenesis: an integrated approach. Mutat Res. 533: 37–65

Roussel C, Neel C, Bril H (2000) Minerals controlling arsenic and lead solubility in an abandoned gold mine tailing. Sci Total Environ 263:209–219

Rzedowski J (2006) Vegetación de México. Fondo de Cultura Económica, México

Sánchez CH, Romero MLA (1992) Mastofauna silvestre del ejido el Limón, municipio de Tepalcingo, Morelos. Universidad Ciencia y Tecnología 2:87–95

Scheirs J, Coan A, Covaci A, Beernaert J, Kayawe M, Caturla M, Wolf H, Baert P, Van Oostveldt P, Verhagen R, Blust R, Coen W (2006) Genotoxicity in wood mice (*Apodemus sylvaticus*) along a pollution gradient: exposure age, and gender-related effects. Environ Toxicol Chem 25:2154–2162

Schneider S, Roessli D, Excoffier L. ARLEQUIN ver 2.0: A software for population genetics data analysis. 2000. Genetics and Biometry Laboratory, University of Geneva, Geneva.

Secretaría de Economía (2011) Panorama Minero del Estado de Morelos. Servicio Geológico Mexicano, serie panorama minero de los estados, México

SEMARNAT. Secretaría de Medio Ambiente y Recursos Naturales (2004) Dirección general del centro nacional de investigación y capacitación ambiental. Dirección de Investigación en Residuos y Proyectos Regionales. Evaluación de tecnologías de remediación para suelos contaminados con metales. Tania L. Volke Sepúlveda J. Antonio Velasco Trejo, D. Alejandro de la Rosa Pérez, Gustavo Solórzano Ochoa. Etapa I

SEMARNAT. Secretaría de Medio Ambiente y Recursos Naturales (2005) Dirección de Investigación en Residuos y Sitios Contaminados. Subdirección de Investigación en Sitios Contaminados y Sustancias Tóxicas. Informe anual de actividades. Evaluación de tecnologías de remediación para suelos contaminados con metales. Etapa II.

Sheffield SR, Sawicka-Kapusta K, Cohen JB, Rattner BA (2001) Rodentia and Lagomorpha. En: Shore RF, Rattner BA (eds), Ecotoxicology of wild mammals. John Wiley and Sons, New York, Pp 215–314

Shore RF, Douben PE (1994) Predicting ecotoxicological impacts of environmental contaminants on terrestrial small mammals. Rev Environ Contam T 134:49–89

Shugart L, Theodorakis C (1998) New trends in biological monitoring: application of biomarkers to genetic ecotoxicology. Biotherapy 11:119–127

Slatkin M (1985) Gene flow in natural populations. Ann Rev Ecol Syst 16:393–430

Slatkin M, Barton N (1989) A comparison of three indirect methods for estimating average levels of gene flow. Evol 43:1349–1368

Smith G, Rongstad O (1982) Small mammal heavy metal concentrations from mined and control sites. Environ Poll 28:121–134

Smith PN, Cobba GP, Harper FM, Adair BM, McMurry ST (2002) Comparison of white-footed mice and rice rats as biomonitors of polychlorinated biphenyl and metal contamination. Environ Pollut 119:261–268

Sneath PHA, Sokal RR (1973) Numerical Taxonomy. The Principles and Practice of Numerical Classification. W. H. Freeman, San Francisco, 573 p

Sommer S (2003) Effects of habitat fragmentation and changes of dispersal behavior after a recent population decline on the genetic variability of noncoding and coding DNA of a monogamous Malagasy rodent. Mol Ecol. 12:2845–2851

Staton J, Schizas N, Chandler G, Coull B, Quattro J (2001) Ecotoxicology and population genetics: The emergence of "phylogeographic and evolutionary ecotoxicology". Ecotoxicology 10:217–222

Statsoft (2000) Statistica for Windows, v. 5.1. Computer program manual. Tulsa, StatSoft Inc

Stockley P, Searle JB, Macdonald DW, Jones CS (1993) Female multiple mating behavior in the common shrew as a strategy to reduce inbreeding. Proc R Soc Lond B 254:173–179

Storelli M, Marcotrigiano G (2005) Bioindicator organisms: Heavy metal pollution evaluation in the ionian sea (Mediterranean Sea-Italy). Environ Monit Assess 102:159–166

Sues B, Tarashewski H, Rydlo M (1997) Intestinal fish parasites as heavy metal bioindicators: A comparison between Acanthocephalus lucci and zebra mussel, Dressena polymorpha. Bull Environ Contam Toxicol 59:14–21

Sunderberg J, Okarsson A (1992) Placental and lactational transfer of mercury from rats exposed to methylmercury in their diet: Speciation of mercury in the offspring. J Trace Elem Exp Med 5:47–56

Swofford DL, Olsen GJ (1990) Phylogeny reconstruction. En: Hillis DM, Moritz C (Eds) Molecular Systematics. Sinauer associated Inc. Massachusetts. Pp 411–501

Talmage S, Walton B (1991) Small mammals as monitors of environmental contaminants. Rev Environ Contam Toxicol 119:47–145

The Mineral Database-mindat.org. 2004. Mineralogía de Huautla, Municipio de Tlaquiltenango, Morelos. *http://www.mindat.org/index.php*

Theodorakis C (2001) Integration of genotoxic and population genetic endpoints in biomonitoring and risk assessment. Ecotoxicology 10:245–256

Tovar-Sánchez E, Cervantes LT, Martínez C, Rojas E, Valverde M, Ortiz-Hernández ML, Mussali-Galante P (2012) Comparison of two wild rodent species as sentinels of environmental contamination by mine tailings. Environ Sci Pollut Res. 19:1677–1686

Tremblay A, Lesbarreres D, Merritt T, Wilson C (2008) Genetic structure and phenotypic plasticity of yellow perch (*Perca flavescens*) populations influences by habitat, predation, and contamination gradients. Integr Environ Assess Manag 4:264–266

Ungherese G, Mengoni A, Somigli S, Baroni D, Focardi S, Ugolini A (2010) Relationship between heavy metals pollution and genetic diversity in Mediterranean population of the sandhopper *Talitrus saltator* (Montagu) (Crustaceae, Amphipoda). Environ Pollut 158:1638–1643

Valavanidis A, Vlachogianni T, Fiotakis C (2009) 8-hydroxy-2-deoxyguanosine (8-OHdG): a critical biomarker of oxidative stress and carcinogenesis. J Environ Sci Health Part C. 27:120–139

Valavanidis A, Vlachogianni T (2010) Metal Pollution in Ecosystems. Ecotoxicology studies and risk assessment in the marine environment. Sci Adv Environ Toxicol Ecotox issues. www.chem-tox-ecotox.org

Valko M, Morris, Cronin (2005) Metals, toxicity and oxidative stress. Curr Med Chem. 12:1161–1208

Valko M, Rhodes C, Moncol J, Izakovic M, Mazura M (2006) Free radicals, metals and antioxidants in oxidative stress-induced cancer. Chem-Biol Interact. 160:1–40

Vanden-Heuvel J, Davis J (1999) Molecular approaches to identify exposure and risk to specific environmental pollutants. Biomarkers 4:93–105

Van de Zande L, Van Apeldoorn RC, Blijdenstein AF, De Jong D, Van Delden W, Bijlsma R (2000) Microsatellite analysis of population structure and genetic differentiation within and between population of the root vole, *Microtus oeconomus* in the Netherlands. Mol Ecol 9:1651–1656

Van Straalen N (1999) Genetic biodiversity in toxicant-stressed populations. Prog Environ Sci 1:195–201

Van Straalen N, Timmermans M (2002) Genetic variation in toxicant-stressed populations: An evaluation of the "genetic erosion" hypothesis. Hum Ecol Risk Assess 8:983–1002

Vargas V (2010) Estructura genética del roedor *Baiomys musculus* (Muridae) en la selva seca caducifolia en el estado de Morelos. Tesis de Licenciatura. Universidad Autónoma del Estado de Morelos

Vega Faúndez A (1999) Minería y Medio Ambiente. Ministerio de educación. Buenos Aires, Argentina.

Volke ST, Velasco TA, De la Rosa PA, Solórzano OG (2004) Evaluación de tecnologías de remediación para suelos contaminados con metales. Etapa I. Secretaría de Medio Ambiente y Recursos Naturales, México

Volke ST, Velasco TA, De la Rosa PA, Solórzano OG (2005) Evaluación de tecnologías de remediación para suelos contaminados con metales. Etapa II. Secretaría de Medio Ambiente y Recursos Naturales, México

Vucetich LM, Vucetich JA, Cleckner LB, Gorski PR, Peterson RO (2001) Mercury concentrations in deer mouse (*Peromyscus maniculatus*) tissues from Isle Royale National Park. Environ Pollut 114:11–38

Weber JN, Peters M, Tsyusko O, Linnen C, Hagen C, Schable N, Tuberville T, Mckee A, Lance S, Jones K, Fisher H, Dewey M, Hoekstra H, Glenn C (2010) Five Hundred Microsatellite Loci for *Peromyscus*. Conserv Genet 11:1243–1246

Weir BS (1996) Genetic Data Analysis II: Methods for Discrete Population Genetic Data. Sinauer Associated Inc. Massachusetts

Weng L, Temminghoff EJ, Van Riemsdijk WH, (2001) Contribution of individual sorbents to the control of heavy metal activity in sandy soil. Environ Sci Technol 35:4436–43

Werre F, Ortiz-Hernández L (2000) Monografía geológica-minera del estado de Morelos. Consejo de Recursos Minerales, México

Wilson D, Thompson L (2007) Molecular mechanisms of sister-chromatid exchange. Mutat Res. 616:11–23

Whitlock M, McCauley D (1999) Indirect measures of gene flow and migration: F_{ST} not equal to 1/(4Nm + 1). Heredity 82:117–25

WHO. World Health Organization (2007) Health risks of heavy metals from long range transboundary air pollution, WHO Regional Office for Europe, Copenhagen

Yap C K, Tan SG, Ismail A, Omar H (2004) Allozyme polymorphisms and heavy metal levels in the green-lipped mussel *Perna viridis* (Linnaeus) collected from contaminated and uncontaminated sites in Malaysia. Environ Inter 30:39–46

Yap CK, Chua BH, Teh CH, Tan SG, Ismail A (2007) Primers of RAPD markers and heavy metal concentrations in *Perna viridis* (L.), collected from metal-contaminated and uncontaminated coastal waters: Are they correlated with each other? Russian J Genet 43:544–550

Yap CK, Chong CM, Tan SG (2011) Allozyme polymorphism in the horseshoe crabs *Carcinoscorpius rotundicauda* collected from polluted intertidal area in Peninsular Malaysia. Environ Monit Assess 174:389–400

Yauk C, Quinn J (1996) Multilocus DNA fingerprinting reveals high rate of heritable genetic mutation in herring gulls nesting in an industrialized urban site. Proc Natl Acad Sci USA 93:12137–12141

Yauk C, Fox G, McCarry B, Quinn J (2000) Induced minisatellite germline mutations in herring gulls (*Larus argentatus*) living near steel mills. Mutat Res 452:211–218

Yeh FC, Yang R, Boyle T (1999) PopGene Ver. 1.31. University of Alberta, Canada

Zar J (2010) Biostatistical analysis. Prentice-Hall, New-Jersey

APÉNDICE A

Environ Sci Pollut Res
DOI 10.1007/s11356-012-1263-8

RESEARCH ARTICLE

Evidence of population genetic effects in *Peromyscus melanophrys* chronically exposed to mine tailings in Morelos, Mexico

Patricia Mussali-Galante · Efraín Tovar-Sánchez · Mahara Valverde · Leticia Valencia-Cuevas · E. Rojas

Received: 16 August 2012 / Accepted: 11 October 2012

Abstract Effects of environmental chemical pollution can be observed at all levels of biological organization. At the population level, genetic structure and diversity may be affected by exposure to metal contamination. This study was conducted in Huautla, Morelos, Mexico in a mining district where the main contaminants are lead and arsenic. *Peromyscus melanophrys* is a small mammal species that inhabits Huautla mine tailings and has been considered as a sentinel species. Metal bioaccumulation levels were examined by inductively coupled plasma mass spectrometry and genetic analyses were performed using eight microsatellite loci in 100 *P. melanophrys* individuals from 3 mine tailings and 2 control sites. The effect of metal bioaccumulation levels on genetic parameters (population and individual genetic diversity, genetic structure) was analyzed. We found a tissue concentration gradient for each metal and for the bioaccumulation index. The highest values of genetic differentiation (*Fst* and *Rst*) and the lowest number of migrants per generation (*Nm*) were registered among the exposed populations. Genetic distance analyses showed that the most polluted population was the most genetically distant among the five populations examined. Moreover, a negative and significant relationship was detected between genetic diversity (expected heterozygosity and internal relatedness) and each metal concentration and for the bioaccumulation index in *P. melanophrys*. This study highlights that metal stress is a major factor affecting the distribution and genetic diversity levels of *P. melanophrys* populations living inside mine tailings. We suggest the use of genetic population changes at microgeographical scales as a population level biomarker.

Keywords Small mammals · *Peromyscus melanophrys* · Metals · Mine tailings · Genetic diversity · Genetic structure · Bioaccumulation

Responsible editor: Stuart Simpson

P. Mussali-Galante · M. Valverde · E. Rojas (✉)
Departamento de Medicina Genómica y Toxicología Ambiental,
Instituto de Investigaciones Biomédicas,
Universidad Nacional Autónoma de México,
México, D.F. C.P. 04510, Mexico
e-mail: emilior@biomedicas.unam.mx

E. Tovar-Sánchez · L. Valencia-Cuevas
Departamento de Sistemática y Evolución,
Centro de Investigación en Biodiversidad y Conservación,
Universidad Autónoma del Estado de Morelos,
Av. Universidad 1001, Col. Chamilpa, Cuernavaca,
Morelos CP 62210, Mexico

Published online: 30 October 2012

Introduction

The environment is continuously loaded with foreign chemical substances, released by anthropogenic activities. Depending on their persistence and potential toxicity numerous contaminants are suspected to cause adverse effects across levels of biological organization. Among environmental pollutants, metals have been identified among the most toxic elements to nearly all living organisms (EPA 2000; WHO 2007). Mining activities, including garbage and sewage sludge disposal in field sites, have introduced several metals into the environment. Moreover, mine tailings and acid mine drainage are sources of major environmental metal pollution to soil, water, and biota living around tailing sites (Jiang et al. 2011). Hence, environmental metal exposure can affect natural populations in many ways, being genetic change as one of the most important alterations that may disrupt genetic equilibrium at all levels of biological organization (Morgan et al. 2007; Bickham 2011).

Metals may affect directly or indirectly the genetic pool of populations. Directly, through their diverse mechanisms of mutagenesis in somatic and germ cells or indirectly, through ecological mediated processes such as demographic

declines, alteration in migration rates, bottlenecks, or selection (Berckmoes et al. 2005). Hence, genetic patterns of wild populations are used to asses and monitor environmental impacts in natural systems (Belfiore and Anderson 1998, 2001; Bickham 2011).

Studies at the molecular, cellular, physiological, and behavioral levels have been used successfully with different animal species for analyzing exposure to contaminants (Bervoets and Blust 2003; Ricketts et al. 2004; Klaper et al. 2006; Brooks et al. 2009). These approaches provide important information about mechanisms of action and dose–response relationships between contaminants and changes at the molecular or individual level. However, studies of effects at the population level which can generate information about the relationship between contaminant exposure and ecological responses are few, particularly in the case of metal contamination in wild small mammal populations (Sheffield et al. 2001; Phelps and McBee 2009).

In order to establish links between pollution effects and population level responses, the utilization of sentinel organisms or bioindicators is an important step in ecotoxicological surveys. Sentinel organisms are a set of taxa that could be utilized to survey locales for the increased presence of mutation stressors (Rogstad et al. 2003). In this context, small mammals are considered as sentinel organisms due to their great abundance, widespread distribution, and short dispersal distances, are in close contact with contaminants due to ingestion and inhalation of contaminated soils, easy capture, and short-life span (Levengood and Heske 2008).

Studies that investigate the relationship between metal exposure and population level effects use molecular markers to analyze changes in genetic structure and diversity of the exposed populations. Among these, microsatellites (SSRs) are the most used because they are highly polymorphic and abundant; they can easily be amplified by PCR and because of their high variability and rapid evolution, they are well suited for studies analyzing recent or rapid changes in genetic variability within populations such as those caused by environmental toxins. Another advantage of using SSR's is the possibility to target multiple loci, thereby increasing the chances to detect mutational events, a fact that is particularly important when monitoring genetic effects in exposed populations (Bickham et al. 2000; Berckmoes et al. 2005; Arif and Khan 2009).

This study was carried out in Huautla, Morelos state, Mexico. Huautla is a mining district known for its historic mining activity since the sixteenth century until 1988, especially lead, silver, and zinc. There were four mines in the region, none of which are active today. It is estimated that there are about 780,000 t of toxic wastes in the zone, the majority of them rich in lead (Pb), manganese (Mn), cadmium (Cd), and arsenic (As) that have not been processed or neutralized (Volke et al. 2005). As a consequence of mining activities, three mine tailings were left in open air and near streams that disembogue at the Amacuzac river. It is estimated that the main contaminants of soil and ground waters are arsenic and lead (Volke et al. 2004, 2005). Also, previous studies (Mussali-Galante 2008) have documented genotoxic effects in Huautla settlers living near mine tailings and in small mammals (*Peromyscus melanophrys* and *Biomys musculus*; Tovar-Sánchez et al. 2012) populations living inside these mine tailings. Therefore, the aims of this study were: (a) to analyze bioaccumulation levels in *P. melanophrys* individuals from three exposed and two reference sites and (b) to investigate if *P. melanophrys* populations inhabiting inside mine tailings show altered patterns of genetic structure and diversity in comparison to reference populations, using microsatellite markers.

Materials and methods

Study sites

All study sites are located southwest of the municipality of Tlaquiltenango, in a protected natural reserve, known as "REBIOSH" (Reserva de la Biosfera Sierra de Huautla) in Morelos state, Mexico, except from control 1 (Werre and Ortiz-Hernández 2000; Volke et al. 2004; Dorado et al. 2005; Fig. 1).

Control sites Ajuchitlán (control 1) is located at 18°27′ N–98°58′ W, in an altitude of 1.060 m Quilamula (control 2) is located at 18°30′ N–98°59′ W, in an altitude of 1.070 m (INEGI 2004, 2009). Both sites are surrounded by deciduous forest (Rzedowski 2006). The control sites were chosen because there are no mines in or near these areas and there are no records of any possible anthropogenic contamination by metals in these zones. Additionally, the water streams and predominant winds flow in a north–south direction (Morales and Carrillo 2010). Therefore, neither the winds nor the watercourses can be dispersing the contaminants from the mine tailings to the control sites. Also, ecological and geographical characteristics are very similar to the exposed sites (Tovar-Sánchez et al. 2012). Moreover, we avoided agricultural activity at the sampling sites in order to evade any agrochemical contamination as a confounding factor. The reference sites are located at 7.50±1.53 linear kilometers from the exposed sites (Fig. 1).

Exposed sites The exposed sites are well-known for their historic mining activity. Mines from this region exploited primarily metal ore deposits of lead, silver, and zinc. During mining activities, three tailings were formed which were put in open air without any environmental care. Therefore, all

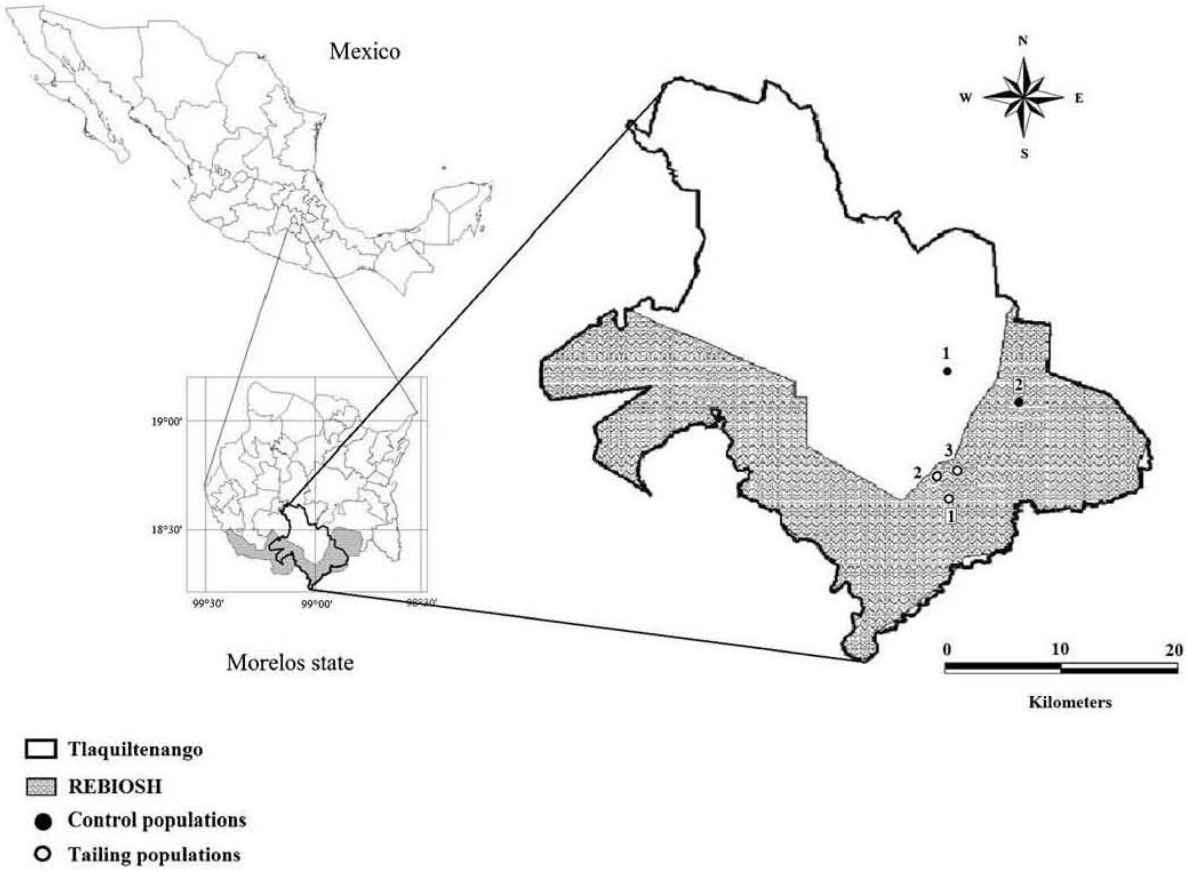

Fig. 1 Map of the geographical locations of the sampled *P. melanophrys* populations in Huautla, at the municipality of Tlaquiltenango, Morelos, Mexico

the resulting mine tailing impoundments have comparable chemical composition and were handled similarly. It is estimated that there are about 780,000 t of toxic wastes in the zone, the majority of them rich in Pb, Mn, Cd, and As, that have not been processed or neutralized. The principal pollutants of the region are lead (2,298 mg/kg) and arsenic (139 mg/kg; Volke et al. 2005). *Mine tailing 1* is the smallest in the zone and it is located at 18°25′40.60″ N 99°01′57.82″ W. *Mine tailing 2* is the intermediate size between tailing 1 and 3 and it is located at 18°26′22.62″ N 99°01′51.71″ W. *Mine tailing 3* is the biggest in the zone and it is located at 18°26′36.37″ N 99°01′26.71″ W, at 500 m from Huautla town. Tailing 3 is the most studied impoundment in terms of its physico-chemical properties and metal content. It is surrounded by deciduous forest (Rzedowski 2006), has a pH of 8.2, a cation exchange capacity of 30.1 cmol(+)/kg, the predominant particulate size is <45 μm (44.2 %), being this fraction where the highest metal concentrations are contained [As (31.9 %), Cd (26.0 %), Pb (30.7 %), and V (29.1 %)] (Volke et al. 2004, 2005).

Study species

P. melanophrys (Coues 1874) is considered as endemic species to Mexico; it occurs from Durango, Coahuila, Nuevo León, and Tamaulipas to Chiapas State in Mexico. It is present in deciduous forests, being more common in arid zones than in temperate mountain regions (Sánchez and Romero 1992). It distributes all across Morelos state, occurring from 100 to 2,600 m a.s.l. (Carleton and Musser 2005). These small mammals are nocturnal and mostly herbivorous (Sánchez and Romero 1992). *P. melanophrys* is the most abundant small mammal at the study sites, which occurs year-round.

Animal sampling

P. melanophrys individuals were sampled from the three mine tailings and from two reference sites. Mice were captured alive with Sherman traps which were baited with oatmeal balls, vanilla, and peanut butter. We established four permanent grids of 100×20 m inside each mine tailing.

A total of 400 Sherman live traps were put separated by 5 m between them, in each site. Trapping was carried out in three consecutive days each week until 20 mice were caught at each mine tailing and reference sites. All the sampling procedures were done in the same season (from August to September 2010) to avoid seasonal fluctuations in density of the sampled individuals. Specimens were handled with clean latex gloves and live specimens were euthanized by cervical dislocation. Necropsies were carried out in order to obtain liver and kidneys. Specimens were identified to species, according to Hall (1981) and classified by sex and age (juveniles, sub-adults or adults, based on pelage, Layne 1968), measured (total length, tail length, cephalic perimeter, hind foot, and ear length) using an automated Vernier scale and weighed. Kidneys were removed and placed in acid-washed microcentrifuge tubes and stored at −80 °C until metal analyses.

All the procedures were done in accordance to the Mexican regulation about sampling and ethical handling of animal specimens FAUT-0251.

Animal density The density of *P. melanophrys* individuals in all study sites was estimated according to Mares and Ernest (1995).

Metal tissue determination

Five randomly chosen animals from each study site (N=25) were selected for metal analyses. Kidney samples were dried to constant weight and subjected to acid digestion using a Microwave Accelerated Reaction System (CEM® MARS-5) with a 4:1 mixture of HNO_3 65 % and HCl 37 % (JT Baker) in closed Teflon bombs. The sample was solubilized and dissolved in distilled water and filtered; this solution was diluted to a final volume of 50 ml until analysis. A sample without tissue was processed simultaneously which was used as a control. Thereafter, metals (Al, Pb, Cu, As, Cd, Hg, Ni) were determined by inductively coupled plasma mass spectrometry (ICP-MS) using an ICP-820 plasma mass spectrophotometer (ICP-MS Systems, Bruker, MA, USA). The instrument was calibrated with standard solutions containing known concentrations of each element. Standard Reference Material of National Institute and Technology and internal reference materials were used for precision, quality assurance and control for selected metal measurements. The detection limits for most elements are around the ppt (ng/l) level and below. For each measurement, average values of five replicates were recorded. All metal concentration values are reported as μg/g dry weight.

In the present study, arsenic, cadmium, copper, lead, nickel, aluminum, and mercury were evaluated because they exhibit high concentrations in soils or have potential for uptake and bioaccumulation and genotoxicity.

DNA extraction and microsatellite genotyping

Genomic DNA was obtained from the liver of each animal using the DNA extraction and purification kit (Wizard genomic DNA purification kit, Promega). DNA was quantified by spectrophotometric reading (spectrophotometer Eppendorf, Germany) and gel electrophoresis after ethidium bromide staining. All DNA samples were diluted to a final volume of 30 ng/μl.

A total of 20 primers from the series PM (*Peromyscus maniculatus*), PO (*P. polionotus subgriseus*; Chirhart et al. 2000; Weber et al. 2010), and BW (*P. maniculatus bairdii*; Mullen et al. 2006) were initially tested for the presence of amplification products, reproducibility, and pattern polymorphism in DNA of five animals from different populations. Eight of these primers [PM-103: (CA)22, PM-104: (CA)27, PM-105: (CA)21, PM-107: (CA)18, PM-109: (CA)25, PM-111: (CA)23, PM-112: (CA)20] yielded the most successful results and were used for the analysis of all samples.

SSRs amplification was carried out in a total volume of 15 μl, containing 30 ng of template DNA, 1 U of Taq DNA polymerase (GoTaq, Promega), 1.5 μM of fluorescently labeled forward and non-label reverse primer, 0.2 mM of each dNTP, 2.5 mM $MgCl_2$, and 1× concentration DNA polymerase buffer (GoTaq, Promega). PCR reaction conditions were as follows: 3 min at 95 °C, followed by 35 cycles composed of 95 °C for 30 s, 30 s at 53 °C, and 25 s at 72 °C. Finally, an extension step at 72 °C for 5 min. Amplification products from all 100 individuals were resolved in capillary electrophoresis on an automated capillary genetic analyzer ABI-3100 (Applied Biosystems, CA, USA) using gene scan ROX-2500 (Applied Biosystems CA, USA) as size standard. Alleles were scored using the Gene Mapper ver. 3.7 Software (Applied Biosystems, CA, USA).

Data analyses

Metal bioaccumulation Two-factor ANOVAs were conducted (model I fixed effects; Zar 2010) to determine the effect of the study site, individual, and interaction site× individual on tissue metal concentrations (Al, Pb, Cu, As, and Cd) in *P. melanophrys*. Also, a Tukey's honestly significance difference test was conducted to detect significant differences between each mean metal concentration registered among study sites (Zar 2010).

We calculated a relative bioaccumulation index of individuals per site, by dividing the individual concentration of the metal i (C_i) by the total observed concentration (C_{iTotal}) and averaging over all metals, to relate metal

bioaccumulation of individuals per site and genetic diversity. Thus, the individual mean (multi-metal) bioaccumulation index (IMBI) was defined as:

$$IMBI = \frac{\sum_{i-1}^{n} C_i / C_{itotal}}{n}$$

with n the total number of metals, C_i the individual concentration of the metal i, C_{iTotal} the total concentration of the metal i. IMBI ranges from 0 to 1 (modified from Maes et al. 2005). To compare metal bioaccumulation in *P. melanophrys* individuals among study sites, a two-factor ANOVA (model I fixed effects; Zar 2010) analysis was performed on the IMBI values, followed by post hoc analyses (Tukey tests). All statistical analyses were done with the software STATISTICA 6.0 (StatSoft 2000).

Animal density One-way analysis of variance (ANOVA) was conducted in order to evaluate the effect of the site on *P. melanophrys* density. Also, a multiple comparison analysis test (Tukey) was used to determine significant differences between the mean density values among sites. Data were log transformed (X'=log X+1; Zar 2010). Density estimates are given in individuals/hectare (ind./ha).

Genetic analyses

A) *Genetic structure:* Population structure for *P. melanophrys* was estimated using an analysis of molecular variance (AMOVA; Excoffier et al. 1992) to test how sequence variation is partitioned within and among populations at all sites. Genetic differentiation indices were calculated for both mutational models [stepwise (*Rst*) and infinite allele (*Fst*)] (Nei 1978).
B) *Genetic distance:* Population relationships were inferred using the UPGMA clustering method on the basis of Nei (1978) unbiased genetic distance with POPGENE (Sneath and Sokal 1973; Swofford and Olsen 1990). The tree was subsequently visualized with TREEVIEW version 1.52 (Page 1996). Significance tests of correlations were performed by bootstrapping over loci with a 95 % nominal confidence interval (Weir 1996).
C) *Genetic diversity:* We estimated the genetic diversity of *P. melanophrys* using allele frequencies per locus in each population, using eight nuclear loci (SSR). Also, we pooled all loci to obtain the mean number of alleles (*A*) and average expected heterozygosity (*He*). Genetic data were analyzed with TFPGA v. 1.3 (Miller 2000) and POPGENE v. 1.31 (Yeh et al. 1999).

A non-parametrical analysis of variance (Kruskal–Wallis) was used to determine significant differences among average expected heterocigozity (*He*) of *P. melanophrys* studied populations. Also, a multiple comparison analysis test (Tukey) was conducted to determine significant differences between the mean values of *He* of all the studied populations (Zar 2010).

Individual genetic diversity of *P. melanophrys* individuals was quantified using the internal relatedness (IR) parameter (Amos et al. 2001) with eight microsatellite loci. IR is based on the relatedness measure of Queller and Goodnight (1989), except that at each locus, two alleles rather than two pairs of alleles are compared. Over several loci, the resulting values are approximately normally distributed and centered on zero, with negative values suggesting relatively outbred individuals and high positive values being suggestive of inbreeding. IR values were computed using EXCEL (Microsoft INC.) macro written in Visual Basic provided on the William Amos website (http://www.zoo.cam.ac.uk/zoostaff//meg/amos.htm, Department of Zoology, Cambridge University, UK). For this study, IR values were multiplied by (−). Thus, IR values below zero denote inbreed individuals and values above zero denote outbred individuals.

In this study, genetic diversity parameters (*A* vs. *He*) were significantly related between them (r=0.090, P=0.01). Therefore, regression analyses were conducted in order to investigate the relationship between the genetic diversity (*He* and IR) and metal bioaccumulation (Al, Pb, Cu, As, Cd, and IMBI), in *P. melanophrys* individuals of the studied populations. Expected heterozygosity and each metal concentration data were log-transformed and IMBI values were corrected as X=arcsin (%)1/2 (Zar 2010).

Results

Metal bioaccumulation From the total of metals analyzed (Al, Pb, Cu, As, Cd, Hg, and Ni), Hg and Ni were not detected in any tissue sample. The same metal mixture was registered in *P. melanophrys* kidneys from all study sites. In general, analyses of variance (ANOVA) detected a significant effect of the site (S), of the individual (I) and of the interaction S×I on each metal concentration and for the IMBI in *P. melanophrys*. In general, Tukey's test showed that metal tissue concentrations did not differ significantly between individuals from both control sites. In contrast, metal concentrations in individuals from the control sites differed significantly from all exposed sites (Table 1).

The distribution of metal concentrations between sites was heterogeneous, as shown in Fig. 2. The distribution of the IMBI values ranged from 0.07 to 0.43 (Fig. 2). Moreover, a metal tissue concentration gradient for each metal and for the IMBI was found in *P. melanophrys*: control 1= control 2<tailing 1<tailing 2<tailing 3 (Table 1).

Density of P. melanophrys A significant effect of the site on *P. melanophrys* density was registered ($F_{4,40}$=10.437, P<0.01).

Table 1 Mean metal concentrations (μg/g DW) and standard deviation in kidneys of *Peromyscus melanophrys* from the control and exposed sites (tailings)

Site	*N*	Al	Pb	Cu	As	Cd	IMBI
Control 1	5	23.2±3.6 a	0.9±0.2 a	6.8±1.1 a	0.1±0.1 a	0.1±0.1 a	0.1±0.0 a
Control 2	5	22.4±5.6 a	0.8±0.3 a	7.1±2.0 a	0.2±0.1 a	0.1±0.1 a	0.1±0.0 a
Tailing 1	5	35.6±11.2 b	3.3±0.6 b	11.6±0.4 b	0.5±0.1 b	1.1±0.2 b	0.2±0.0 b
Tailing 2	5	42.9±19.6 bc	11.9±5.4 c	16.1±3.2 c	0.7±0.2 c	5.4±1.9 c	0.3±0.0 c
Tailing 3	5	46.8±21.5 c	24.3±7.9 d	19.1±5.8 d	1.3±0.5 d	7.1±2.2 d	0.4±0.0 d
ANOVA: Site (S)		$F_{4,100}$=59.181*	$F_{4,100}$=654.073*	$F_{4,100}$=185.921*	$F_{4,100}$=228.562*	$F_{4,100}$=1419.047*	$F_{4,100}$=3010.933*
Individual (I)		$F_{4,100}$=16,451*	$F_{4,100}$=25.187*	$F_{4,100}$=13.794*	$F_{4,100}$=17.534*	$F_{4,100}$=62.302*	$F_{4,100}$=38.877*
S×I		$F_{16,100}$=72.231*	$F_{16,100}$=26.660*	$F_{16,100}$=18.097*	$F_{16,100}$=34.818*	$F_{16,100}$=68.383*	$F_{16,100}$=16.357*

One-way ANOVA results to determine the effect of study sites on metal concentrations are given (aluminium, lead, copper, arsenic, cadmium). Different letters denote significant differences at $P<0.05$ (Turkey's honestly significant difference test)

IMBI individual mean (multi-metal) bioaccumulation index

*$P<0.001$

Also, Tukey analysis ($P< 0.05$) showed the following pattern: Control 2 (11.36 ind./ha)=control 1 (9.34 ind./ha)>tailing 2 (5.56 ind./ha)=tailing 1 (4.04 ind./ha)>tailing 3 (1.52 ind./ha). These results indicated that the lowest density of individuals was registered in mine tailing 3, which was significantly different from mine tailing 1 and 2 and from control sites.

Genetic differentiation among populations Molecular analysis of variance (AMOVA) with the stepwise mutation model showed that the fixation index was statistically different for each site (control=0.39, exposed=0.33). The highest variation was registered within control populations (81.67 %) and the remaining 18.33 % among them. Exposed populations showed the highest genetic variation (62.84 %) between them.

On the other hand, the analysis of molecular variance with the infinite allele model showed that the fixation index was statistically different between control and exposed sites (control=0.59, exposed=0.45). Exposed (41.18) and control populations (9.86) showed the highest genetic variation within populations.

The number of migrants per generation (Nm) was almost six times higher in control populations (5.74) than in exposed populations (0.99; Table 2).

Genetic distance The analysis of genetic distances revealed that both control sites were the most genetically similar. Both control populations shared more genetic similarity with mine tailing 1 population. These populations are more similar to mine tailing 2. Finally, population from mine tailing 3 was the most genetically distant among the five populations examined (Fig. 3).

Genetic diversity All microsatellite loci were highly polymorphic, with the total number of alleles per locus ranging

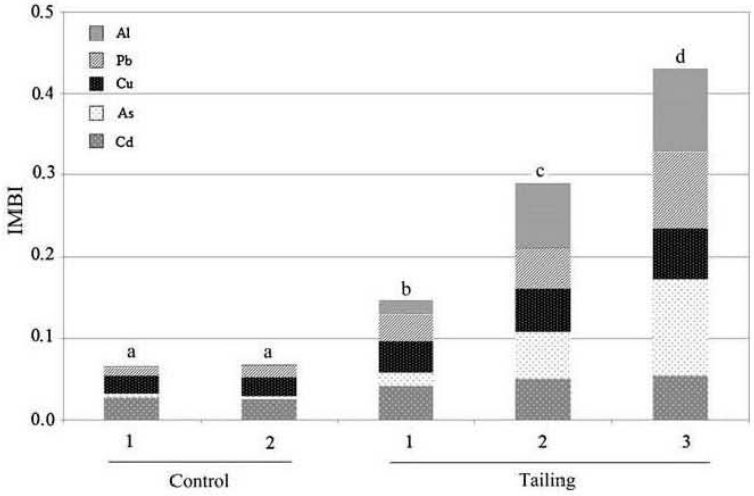

Fig. 2 IMBI [individual mean (multi-metal) bioaccumulation index, modified from Maes et al. 2005] in *P. melanophrys* kidneys in each study site in Morelos, Mexico. Different letters denote significant differences at $P<0.05$ (Tukey's honestly significant difference test)

Table 2 Genetic diversity and genetic structure at nDNA markers in five Mexican population of *Peromyscus melanophrys*

Population	*N*	No. of loci	*A*	*He*	*Nm*	IAM F_{ST}	SMM R_{ST}
Control 1	20	8	11.2	0.786	5.114		
Control 2	20	8	10.7	0.837	6.315		
Mean (±1SD)			11.0±0.4	0.8±0.0	5.7±1.3	0.099*	0.183*
Tailing 1	20	8	9.7	0.643	1.205		
Tailing 2	20	8	9.2	0.605	1.072		
Tailing 3	20	8	8.5	0.421	0.702		
Mean (±1SD)			9.1±0.8	0.6±0.1	1.0±0.1	0.412*	0.628*

N number of individuals, *A* mean number of alleles, *He* expected heterozygosity, *Nm* migrant estimate, F_{ST} and R_{ST} genetic differentiation, *IAM* infinite allele model, *SMM* stepwise mutation model

from 12 to 17. The mean number of alleles was 9.9 and ranged from 8.5 to 11.2. Genetic diversity indexes (*He*) showed the next pattern among sites: control 2 (*He*= 0.786)>control 1 (*He*=0.837)>tailing 1 (*He*=0.643)>tailing 2 (*He*=0.605)>tailing 3 (*He*=0.421). A Kruskal–Wallis analysis of variance showed significant differences in genetic diversity indexes (*He*) among sites (H=19.33, P< 0.001). A multiple comparison Tukey test showed that these indexes were significantly different among sites. *He* values of all studied populations decreased as the metal concentration gradient increased (Table 2).

Relationship between metal concentration and genetic diversity In general, a negative and significant relationship was detected between genetic diversity (*He*) and each metal (Al, Pb, Cu, As, Cd) and multi-metal (IMBI) concentrations in *P. melanophrys*. The coefficient of determination (r^2) ranged from 0.78 for Cd to 0.88 for the IMBI (Table 3, Fig. 4a). Also, the relationship between individual genetic diversity (IR) and each metal and multi-metal (IMBI) concentrations in *P. melanophrys* showed a similar pattern. The coefficient of determination (r^2) ranged from 0.22 for Al to 0.29 for As, Cd, and for the IMBI (Table 3, Fig. 4b).

Discussion

Metal bioaccumulation in *P. melanophrys*

The results of this study showed that *P. melanophrys* populations living inside mine tailings are chronically exposed to a complex metal mixture. Bioaccumulation levels in individuals from the control sites differed significantly from all exposed sites (Table 1). *P. melanophrys* individuals from both control sites had detectable metal levels. We suggest that the metal burdens registered in *P. melanophrys* individuals from control sites are due to an exposure from metals that occur naturally in the soils of this particular region, where a natural richness of mineral soils (mainly sulfur-minerals) of silver and lead has been documented. The main minerals found in this region are: arsenopyrite (FeAsS), galena (PbS), acanthite (Ag_2S), and calclacite (Cu_2S; Volke et al. 2004, 2005; Secretaría de Economía 2011).

The metal bioaccumulation gradient found in this study, describes that among the exposed populations, the less polluted was found in mine tailing 1 and the most polluted was found in mine tailing 3. These results are in accordance to the size and abandonment time of the different mine tailings at Huautla zone (which is inversely related to metal concentration, as a

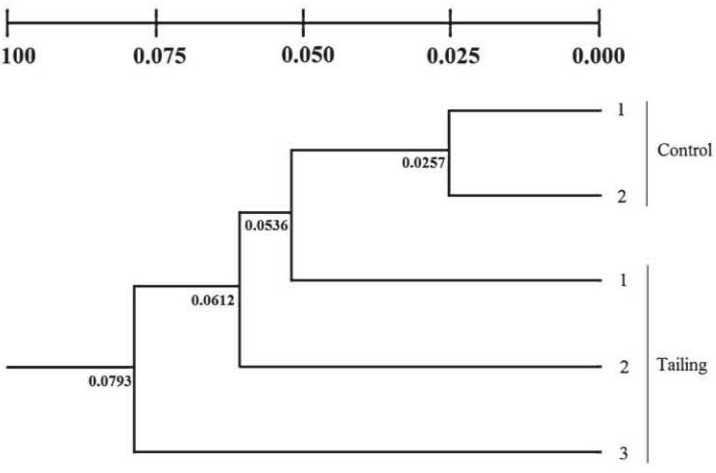

Fig. 3 Unweighted pair-group method using arithmetic average (UPGMA) dendogram of similarity among populations. The dendogram shows the genetic distances among five *P. melanophrys* populations in Morelos, Mexico. The tree was calculated from eight nuclear microsatellite loci

Table 3 Relationship between genetic diversity (He, IR) and each mean metal concentration and the IMBI [individual mean (multi-metal) bioaccumulation index] in *Peromyscus melanophrys* populations from Morelos, Mexico

	r^2	F	P	r^2	F	P
	Aluminium			Lead		
He	0.84	22.911	0.017	0.84	22.911	0.017
IR	0.22	6.428	0.018	0.27	8.579	0.007
	Copper			Arsenic		
He	0.87	27.101	0.014	0.86	25.389	0.015
IR	0.24	7.263	0.013	0.29	9.557	0.005
	Cadmium			IMBI		
He	0.78	16.045	0.027	0.88	35.336	0.009
IR	0.29	9.515	0.005	0.29	9.137	0.005

He expected heterozygosity, *IR* internal relatedness, r^2 coefficient of determination

result of metal leaching and erosion processes). *P. melanophrys* individuals from the biggest and the youngest mine tailing (tailing 3) had the highest bioaccumulation levels, decreasing significantly towards the middle size tailing (tailing 2) and towards the smallest and oldest tailing (tailing 1). Our findings indicated that bioaccumulation levels registered among exposed populations accurately reflect the level of pollution in their immediate environment. Similar findings have been reported in other non-mammalian species (*Anguilla anguilla*, Maes et al. 2005), as well as in other small mammal species (*Apodemus flavicollis*, Folkeson et al. 1990; *Peromyscus maniculatus*, Laurinolli and Bendell-Young 1996; *Apodemus sylvaticus* and *Clethrionomys glareotus*, Erry et al. 2000). However, there are other studies that failed to detect metal bioaccumulation levels in small mammal species from superfund sites (Pascoe et al. 1994; Vucetich et al. 2001). Overall, this study shows that *P. melanophrys* from mine tailing sites can accumulate metals from their environment and supports the recommendation that *P. melanophrys* may serve as a sentinel species for ecotoxicological surveys.

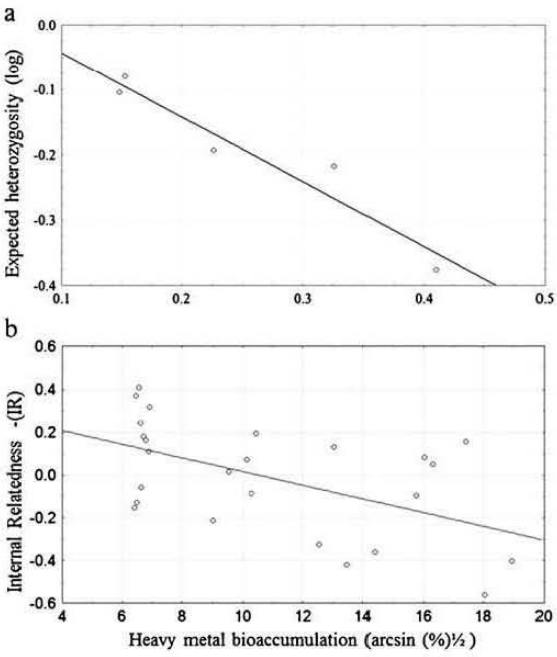

Fig. 4 **a** Relationship between genetic diversity (*He*) and the IMBI [individual mean (multi-metal) bioaccumulation index]. **b** Relationship between individual genetic diversity (IR) and the IMBI [individual mean (multi-metal) bioaccumulation index] in *P. melanophrys* populations in Morelos, Mexico

Density of *P. melanophrys*

In the present study, we registered a significant decrease in *P. melanophrys* density in the most polluted site in comparison to the uncontaminated sites. Our results are in agreement to those reported by Bengtsson et al. (1983) in different species of earthworms (*Deschampsia flexuosa*, *Vaccinium myrtillus*, and *Dendrobaena octaedra*) exposed to metal pollution. Their results showed that the density of earthworms was proportional to the distance from the mill site. Also, *D. octaedra*, the most abundant lumbricid species, was absent in soils within 1 km from the mill. They suggested that the reduced density of earthworms near the mill was due to metal toxicity. Also, for small mammal species, similar results have been reported. For example: Dmowski et al. (1995) showed that the density of the vank vole (*Myodes glareolus*) population in metal contaminated sites was very low in contrast to uncontaminated sites, suggesting the possible effects of metal pollution on nest mortality of suckling individuals. Likewise, Phelps and McBee (2009) observed a decrease in small mammals (*Neotoma floridana* and *Reithrodontomys fulvescens*) density in superfund sites.

These last results highlight the effects of chronic metal exposure on population demographic parameters (i.e., population density). Some authors have shown that pollution can have an influence on the stability of populations by increasing mortality or decreasing reproductive output especially in lead, cadmium, and arsenic exposures (Peakall 1992; Shore and Douben 1994; Burger 1995; Burger and Gochfeld 1996; Dauwe et al. 2004). Hence, reproductive impairment, leading to low fertility, low viability, thus low reproductive success may affect the fitness of the exposed population. Therefore, lower fitness can act as an accelerating factor in reducing the number of individuals in stressed populations (Lande 1988; Bickham et al. 2000; Theodorakis 2001; Medina et al. 2007; Phelps and McBee 2009). Moreover, reduction in group size towards levels below sustainability may drive the group to extinction by mutational meltdown (Lynch et al. 1995).

Metal bioaccumulations vs. genetic structure

In this study, genetic structure estimates for control populations, showed that 10 % (F_{ST}) and 18 % (R_{ST}) of the total genetic variation, was attributed to differences between populations at a micro-geographical scale (7.5 km). Our results are in agreement with other F_{ST} values reported for small mammal species at similar geographical scales, for example, *Spermophilus mollis* (F_{ST}=0.10, 8–13 km, Antolin et al. 2001), *Rattus norvegicus* (F_{ST}=0.07, 0.7–13 km, Gardner-Santana et al. 2009), and *Baiomys musculus* (F_{ST}=0.11, <4 km, Vargas 2010). These results suggest that small mammal populations are genetically structured at micro-geographical scales, mainly because of their limited dispersion rates (Peakall et al. 2003).

In contrast, genetic structure estimates for exposed populations, showed that 41 % (F_{ST}) and 63 % (R_{ST}) of the total genetic variation was attributed to differences between populations. Our results are in accordance to those reported for *Perca flavescens* (θ_{ST}=0.36 and R_{ST}=0.39, Bourret et al. 2008) in a gradient of metal contamination (Cu and Cd), using seven microsatellite loci. However, these results are in contrast to those registered for the rodent *Apodemus sylvaticus* using 10 microsatellite loci, where only 1.58 % of the genetic variability could be attributed to differences between the three most polluted sites versus the four less polluted sites, whereas 6.02 % of the genetic variation was observed among populations within groups in polluted sites (Ag, As, Ad, Cu, and Pb; Berckmoes et al. 2005). We suggest that the high genetic differentiation found in *P. melanophrys* exposed populations could be the result of altered migration rates and low gene flow between them (mean Nm=0.993). In general, these findings are supported by the cluster analysis, showing that the population from mine tailing 3 was the most genetically distant from the control populations. Also, the cluster analysis indicated that the contaminated sites were more similar to one another than to the non-contaminated sites, this result is in line with the findings of Yap et al. (2004, 2011). Hence, genetic distance of *P. melanophrys* populations may serve as a potential genetic indicator for monitoring environmental changes.

It is important to note that genetic structure of *P. melanophrys* exposed populations in this region may be explained on the basis of the pattern of exposure to metals. Thus, the genetic effect of chemical stress in partitioning the genetic variation is a major factor affecting the distribution of genetic variation.

P. melanophrys populations from control sites exhibited almost six migrants per generation. Migration rates are determined by species life history traits, like population density. *P. melanophrys* density in control sites was 10.35 ind./ha, which is similar to other registries of individuals of the genus *Peromyscus* at the REBIOSH (9.18 ind./ha; Cadena 2003). These estimates may be related to higher dispersion rates, as in the case of other small mammal species like *Hypogeonomys antinema* (Sommer 2003) and *Arvicola terrestres* (Berthier et al. 2006).

On the other hand, migration rates in exposed populations were low (0.993) compared with control sites. Low migration rates among chronically exposed populations to metal stress may be the result of lower densities, a fact that is in accordance to the low density registered *P. melanophrys* populations from exposed sites (mean density 3.70 ind./ha). Also, as discussed earlier, ecological processes resulting from the genotoxic effects of metals may lower the average fitness of the population by reducing the number of offspring that contributes to the next generation, resulting in altered migration patterns, which in turn, results in low gene flow and high genetic differentiation rates (Van Straalen 1999; Van Straalen and Timmermans 2002; Maes et al. 2005). Hence, this study demonstrates that metal contamination has altered migration patterns of *P. melanophrys* populations that inhabit Huautla mine tailings.

Metal bioaccumulations vs. genetic diversity

We clearly showed a strong correlation between metal bioaccumulation and the levels of genetic diversity in *P. melanophrys* populations living inside Huautla mine tailings.

In this study, the degree of genetic diversity found in *P. melanophrys* populations from uncontaminated areas (*He*= 0.812), is comparable with the genetic diversity found in other studies on populations of the genus *Peromyscus* (Chirhart et al. 2005, *He* 0.80 to 0.95 in *P. melanossis*; Mossman and Waser 2001, *He*=0.80 in *P. leucopus*). As well as in other small mammal species living in uncontaminated areas (Van de Zande et al. 2000, *He*=0.84 in *Microtus oeconomus*; Berthier et al. 2005, *He*=0.82 in *Arbicola terrestris*, Peakall and Lindenmayer 2006, *He*=0.86 in *Rattus fuscipes*; Gauffre et al. 2008, *He*=0.88 in *Microtus arvalis*).

We registered a negative and significant relationship between genetic diversity (*He*, IR) and bioaccumulation levels (independently of the type of metal or multi-metal bioaccumulation) in *P. melanophrys*. In general, the highest values of the determination coefficient were registered when analyzing the genetic diversity at the population level (*He*) in comparison to the individual level diversity (IR). These findings suggest that the individuals that conform *P. melanophrys* populations present a wide range of genetic diversity (IR; 0.6<0>0.6), registering individuals with IR values close to zero that born to unrelated parents, positive values suggesting relatively outbred individuals and high negative values being suggestive of inbreeding.

Additionally, the wide range of genetic diversity observed when plotting IR values against each metal or the IMBI is possibly the result of inter-individual variation in metal bioaccumulation from the environment (Ma et al.

1991; Smith et al. 2002). First, *P. melanophrys* species burrow in the soil resulting in increased exposure to soil contaminants. Second, mice are exposed to metals directly through their diet (Ma et al. 1991). They feed on seeds that are present mainly in the soil, hence, incidental ingestion and inhalation of contaminated soils with different metal contents (as in the case of heterogeneous distribution of metals along mine tailings, Fig. 2) may be contributing to the observed effects. Also, *P. melanophrys* individuals that live inside Huautla mine tailings feed on plants like *Prosopis laevigata*, *Acacia farneciana*, and *Pitecelobium dulcis*, which have been shown to hiperaccumulate metals (Pb, Cu, Zn) from contaminated soils in Huautla region (unpublished data). Thus, the primary exposure routes for *P. melanophrys* individuals to soil contaminants consist of the consumption of contaminated foods and water, and incidental ingestion and inhalation of soil. Additionally, the variation in individual genetic diversity values may be the result of differences in xenobiotic detoxification metabolism (Smith 2002), gender related effects and of the inter-individual levels of DNA damage observed in these individuals (Tovar-Sánchez et al. 2012).

The loss of genetic diversity levels observed in *P. melanophrys* populations exposed to mine tailings in Huautla zone is similar to other wild animal populations exposed to metal pollution, for example: *Littorina brevicula* (Kim et al. 2003), *Pleurocerca canaliculatum* (Benton et al. 2002), *Rana ridibunda* (Matson et al. 2006), *Hattheyella crassa* (Gardeström et al. 2008), *Pachygrapsus marmoratus* (Fratini et al. 2008), and *Talitrus saltator* (Ungherese et al. 2010). Changes in genetic diversity parameters were mostly attributed to selection of certain genotypes, a reduction of population size and low gene flow between populations. The loss of genetic diversity in populations subjected to anthropogenic stress can be designated as "genetic erosion" and may be considered as a factor of concern in risk assessment of toxic chemicals (Van Straalen and Timmermans 2002). In this context, Fratini et al. (2008) found a decrease in genetic diversity levels in the intertidial crab *Pachygrapsus marmoratus* exposed to a metal mixture (As, Pb, Cd, Cu). Likely results were found by Ungherese et al. (2010) in the amphipod sandhopper *Talitrus saltator* exposed to Hg, Cu and Cd. Both studies suggest that genetic erosion is occurring in these populations and our study supports this hypothesis by showing the negative influences of metal contamination on genetic diversity. However, there are other studies (Berckmoes et al. 2005) where populations of the wood mouse (*Apodemus silvaticus*) from the most metal polluted sites in the gradient did not differ from those of the less polluted sites in terms of heterozygosity levels. Moreover, the authors did not find a correlation between measures of genetic diversity and the degree of metal pollution. They explained that pollution-induced stress was not intense enough, or that insufficient time has passed since the onset of pollution stress (more than 50 years) to induce a population genetic effect. Also, they argue that gene flow masks the negative effects of metal contamination on the population genetic structure. These observations clearly contrast with our results, where the effects of such confounding factors were diminished by the fact that the populations analyzed had very low levels of gene flow (Nm=0.99) and by a reduction in population size (density).

In general, a loss of genetic diversity in populations from mine tailing sites compared with control sites may have a variety of explanations, because metals can exert their toxic effects at all levels of biological organization: At the molecular level, they can alter the DNA molecule, leading to genotoxic insults (DNA adducts, single and double strand breaks, chromosome aberrations, oxidized bases etc.; Scheirs et al. 2006; Bernard 2008; Pra et al. 2008), causing cell and tissue injuries, resulting in health effects at the individual level (Valavanidis and Vlachogianni 2010). In this context, our previous study showed that *P. melanophrys* populations from Huautla mine tailings had significantly higher levels of DNA damage than rodents from control sites. Moreover, we also observed a gender effect, where females had more DNA damage levels than males (Tovar-Sánchez et al. 2012). Genotoxic insults, as a consequence of chronic metal exposure, may alter somatic or germ cells. If alterations arise in the former, health effects on individuals may cause reduced longevity, altering size, and sex proportions of individuals. In contrast, if alterations arise in germ cells, teratogenicity and inheritable alterations may arise, causing reduced reproductive success, low viability, and low fertility, especially if females have higher levels of genotoxic damage, as in the case of Huautla *P. melanophrys* populations. Both scenarios will result in decreased genetic diversity of the exposed population (Bickham et al. 2000). Therefore, metal-related effects can be observed at the population level (Bickham and Smolen 1994; Shugart and Theodorakis 1998; Theodorakis 2001; Medina et al. 2007) where a reduced genetic diversity may be a direct result of exposure to contamination via the process of genotypic selection. Selection may favor tolerant genotypes over intolerant ones, causing population-level genetic changes (Lefèbvre and Vernet 1990; Deng et al. 2007; Yap et al. 2007).

Also, changes in demographic patterns such as low density of the exposed populations may reduce their genetic diversity (Hebert and Murdoch-Luiker 1996; Bickham et al. 2000), as reported by Hebert and Murdoch-Luiker (1996), which analysis on fish populations, revealed a consistent depression of haplotype diversity along with demographic collapses at polluted sites. These results support our findings of low density levels in *P. melanophrys* populations from mine tailings in comparison with populations from both control sites, where the most polluted population had the lowest density and genetic diversity levels.

Altered migration patterns are another cause of reduction in genetic diversity levels in exposed populations, where low migration rates may reduce genetic diversity (Staton et al. 2001; Van Straalen and Timmermans 2002). In this study, *P. melanophrys* control populations exhibited almost six times higher migration rates than exposed populations. According to the one migrant-per-generation rule and to more recent estimates that have suggested that five to ten migrants per generation are required to conserve the genetic diversity of many animal populations (Mills and Allendorf 1996), control populations showed sufficient number of migrants per generation in order to maintain their genetic diversity levels, whereas exposed population showed low levels of gene flow to maintain higher levels of genetic diversity. These findings support the loss of genetic variability in exposed populations, via the reduction in migration rates, lowering gene flow among close populations, which would tend to augment the effect of genetic bottlenecks (Belfiore and Anderson 1998).

All the above mentioned results, may explain the increasing endogamy in the exposed populations (IR), in comparison with control populations where there is gene flow, low endogamy and low genetic differentiation. These observations agree with the suggestion that chemical pollution may reinforce the reproductive isolation of exposed populations where high levels of inbreeding are observed (Nacci and Hoffman 2008; Brown et al. 2009). Our results support the idea that mine tailing sites generally contain high metal levels and might be considered as ecological islands (Deng et al. 2007). Particularly, inbreeding can be pronounced in small wildlife populations that result following bottlenecks caused by pollution incidents (Bickham et al. 2000). In the case of small mammals (i.e., *Sorex araneus*, *Peromyscus leucopus*; Stockley et al. 1993), inbreeding may also be promoted because of their low dispersion rates. This last observation reinforces the use of small mammals as sentinel organisms for evaluating population genetic changes due to pollution events.

Overall, a population that is chronically exposed to metal pollution, that has high levels of DNA damage, reduced density, reduced number of migrants per generation, thus low gene flow, may experience a loss of genetic diversity and high genetic differentiation rates and finally may experience a genetic bottleneck. If so, genetic diversity in exposed populations may be reduced compared to control populations as a consequence of genetic drift (Van Straalen and Timmermans 2002).

Finally, this study supports the "genetic erosion hypothesis" by showing the detrimental effects of metal contamination on genetic diversity in *P. melanophrys* populations.

Conclusions

In wild populations, it is a difficult task to relate population level responses to chemical stress. Despite this, a strong correlation between metal bioaccumulation levels and reduced population genetic diversity in *P. melanophrys* was registered. Moreover, we clearly showed that the genetic effect of chemical stress in partitioning the genetic variation is a major factor affecting the distribution of genetic variation in *P. melanophrys* populations. Therefore, this study is valuable because it reflects the net effect of stressors on small mammal populations and reinforces the idea that genetic structure and diversity may be used as a population level biomarker to elucidate changes in genetic patterns of exposed populations.

Also, this study highlights the suitability of using sentinel organisms like *P. melanophrys* individuals to reveal clear genetic population responses to environmental metal stress using microsatellite markers, despite the geographic proximity of the examined populations.

It is important to mention that when trying to predict population levels responses to environmental stress, the use of biomarkers of internal exposure (metal tissue concentrations) and of early effects (levels of DNA damage) is needed, in order to gain better insights into population genetic responses. Indeed, as the number and type of biomarkers increases, the ability to discriminate between contaminated and non-contaminated related effects also increases. Finally, using microevolutionary changes (loss of genetic diversity) of impacted populations by chemical stress as early warning signs, can be decisive for their preservation—especially if they consist of few individuals—because it allows time for intervention actions and prevent further deterioration of their genetic pool.

Acknowledgments This study was supported by a scolarship to P.M.G. (102684) by the National Council of Science and Technology (CONACyT).This paper constitutes a partial fulfillment of the Graduate Program in Biological Sciences of the National Autonomous University of México (UNAM). The authors thank the "Posgrado en Ciencias Biológicas, Universidad Nacional Autónoma de México (UNAM). We also thank Edith Rivas, Guillermo Sánchez, Evodio Rendon Alquicira, Guadalupe Rangel Altamirano and Laura Márquez for their technical assistance.

References

Amos W, Worthington W, Fullard K, Burger TM, Croxall JP, Bloch D, Coulson T (2001) The influence of parental relatedness on reproductive success. Proc R Soc Lond B 68:2021–2027

Antolin MF, Van Horne B, Berger MD, Holloway AK, Roach JL, Weeks RD (2001) Effective population size and genetic structure of a Piute ground squirrel (*Spermophilus mollis*) population. Can J Zool 79:26–34

Arif I, Khan H (2009) Molecular markers for biodiversity analysis of wildlife animals: a brief review. Animal Biodiv Conserv 32:9–17

Belfiore N, Anderson S (1998) Genetic patterns as a tool for monitoring and assessment of environmental impacts: the example of genetic ecotoxicology. Environ Monit Assess 51:465–479

Belfiore N, Anderson S (2001) Effects of contaminants on genetic patterns in aquatic organisms: a review. Mutat Res 489:97–122

Bengtsson G, Nordström S, Rundgren S (1983) Population density and tissue metal concentration of lumbricids in forest soils near a brass mill. Environ Pollut A 30:87–108

Benton M, Malott M, Trybula J, Dean D, Guttman S (2002) Genetic effects of mercury contamination on aquatic snail populations: allozyme genotypes and DNA strand breakage. Environ Toxicol Chem 21:584–589

Berckmoes V, Scheirs J, Jordaens K, Blust R, Backeljau T, Verhagen R (2005) Effects of environmental pollution on microsatellite DNA diversity in wood mouse (*Apodemus Sylvaticus*) populations. Environ Toxicol Chem 24:2898–2907

Bernard A (2008) Biomarkers of metal toxicity in population studies: research potential and interpretation issues. J Toxicol Environ Health Part A 71:1259–1265

Berthier K, Galan M, Foltete JC, Charbonnel N, Cosson JF (2005) Genetic structure of the cyclic fossorial water voles (*Arvicola terrestris*): landscape and demographic influences. Mol Ecol 14:2861–2871

Berthier K, Charbonnel N, Galan M, Cosson JF (2006) Migration and recovery of the genetic diversity during the increasing density phase in cyclic vole populations. Mol Ecol 15:2665–2676

Bervoets L, Blust R (2003) Metal concentrations in water, sediment and gudgeon (Gobio gobio) from a pollution gradient: relationship with fish condition factor. Environ Pollut 126:9–19

Bickham JW (2011) The four cornerstones of evolutionary toxicology. Ecotoxicology 20:497–502

Bickham J, Smolen M (1994) Somatic and heritable effects of environmental genotoxins and the emergence of evolutionary toxicology. Environ Health Persp 102:25–28

Bickham J, Sandhu S, Hebert P, Chikhi L, Athwal R (2000) Effects of chemical contaminants on genetic diversity in natural populations: implications for biomonitoring and ecotoxicology. Mutat Res 463:33–51

Bourret V, Couture P, Campbell P, Bernatchez L (2008) Evolutionary ecotoxicology of wild yellow perch (*Percha flavescens*) populations chronically exposed to a polymetallic gradient. Aquat Toxicol 86:76–90

Brooks S, Lyon B, Goodsir F, Bignell J, Thain J (2009) Biomarker responses in mussels, an integrated approach to biological effects measurements. J Toxicol Env Health A 72:196–208

Brown AR, Hosken DJ, Balloux F, Bickley LK, LePage G, Owen SF, Hetheridge MJ, Tyler CR (2009) Genetic variation, inbreeding and chemical exposure–combined effects in wildlife and critical considerations for ecotoxicology. Phil Trans R Soc B 364:3377–3390

Burger J (1995) Heavy metal and selenium levels in feathers of herring gulls (*Larus argentatus*): differences due to year, gender, and age at Captree, Long Island. Environ Monit Assess 38:37–50

Burger J, Gochfeld M (1996) Heavy metal and selenium levels in Franklin's gull (*Larus pipixcan*): parents and their eggs. Arch Environ Contam Toxicol 30:487–491

Cadena M (2003) Efecto de la perturbación y estacionalidad en la comunidad de roedores en una salva baja caducifolia en Morelos, México. Dissertation. Universidad de las Américas de Puebla.

Carleton MD, Musser GG (2005) Order Rodentia. In: Wilson DE, Reeder DM (eds) Mammal species of the world a taxonomic and geographic reference. Johns Hopkins University Press, Baltimore, pp 894–1531

Chirhart S, Honeycutt R, Greenbaum I (2000) Microsatellite markers for the deer mouse *Peromyscus maniculatus*. Mol Ecol 9:1661–1686

Chirhart S, Honeycutt R, Greenbaum I (2005) Microsatellite variation and evolution in the *Peromyscus maniculatus* species group. Molec Phylogenet Evol 34:408–415

Coues E (1874) Synopsis of the Muridae in North America. Proc Acad Nat Sci Phila 3:173–196

Dauwe T, Janssens E, Kempenaers B, Eens M (2004) The effect of heavy metal exposure on egg size, eggshell thickness and the number of spermatozoa in blue tit Parus caeruleus eggs. Environ Pollut 129:125–129

Deng J, Liao B, Ye M, Deng D, Lan C, Shu W (2007) The effects of heavy metal pollution on genetic diversity in zinc/cadmium hyperaccumulator *Sedum alfredii* populations. Plant Soil 297:83–92

Dmowski K, Kozakiewicz M, Kozakiewicz A (1995) Ecological effects of heavy metal pollution (Pb, Cd, Zn) on small mammal populations and communities. B Pol Acad Biol Sci 43:1–10

Dorado O, Maldonado B, Arias D, Sorani V, Ramírez R, Leyva E (2005) Programa de conservación y manejo Reserva de la Biosfera Sierra de Huautla. Comisión Nacional de Áreas Naturales. Protegidas, México

EPA Environmental Protection Agency (2000). Innovative remediation technologies: Field scale demonstration projects in North America

Erry BV, Macnair MR, Meharg AA, Shore RF (2000) Arsenic contamination in wood mice (*Apodemus sylvaticus*) and bank voles (*Clethrionomys glareolus*) on abandoned mine sites in southwest Britain. Environ Pollut 110:179–187

Excoffier L, Smouse PE, Quattro JM (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131:479–491

Folkeson L, Nyholm HEI, Tyler G (1990) Influence of acidity and other soil properties on metal concentrations in forest plants and animals. Sci Total Environ 96:211–233

Fratini S, Zane L, Ragionieri L, Vannini M, Cannicc S (2008) Relationship between heavy metal accumulation and genetic variability decrease in the intertidial crab *Pachygrapsus marmoratus* (Decapoda; Grapsidae). Estuar Coast Shelf S 79:679–686

Gardeström J, Dahl U, Kotsalainen O, Maxson A, Elfwing T, Grahn M, Bengtsson B, Breitholtz M (2008) Evidence of population genetic effects of long-term exposure to contaminated sediments: a multi-endpoint study with copepods. Aquat Toxicol 86:426–436

Gardner-Santana LC, Norris DE, Fornadel CM, Hinson ER, Klein SL, Glass GE (2009) Comensal ecology, urban landscapes and their influence on the genetic characteristics of city-dwelling Norway rats (*Rattus norvegicus*). Mol Ecol 18:2766–2778

Gauffre B, Estoup A, Bretagnolle V, Cosson F (2008) Spatial correlation structure of a small rodent in heterogeneous landscape. Mol Ecol 17:4619–4629

Hall ER (1981) The mammals of North America. John Wiley and Sons, New York

Hebert PM, Murdoch-Luiker M (1996) Genetic effects of contaminant exposure-towards an assessment of impacts on animal populations. Sci Total Environ 191:23–58

INEGI (2004) Instituto Nacional de Estadística y Geografía. Información Geográfica del Estado de. Morelos, México

INEGI (2009) Instituto Nacional de Estadística y Geografía. Información Geográfica del Estado de. Morelos, México

Jiang ZF, HuangSZ HYL, Zhao JZ, Fu JJ (2011) Physiological response of Cu mine tailing remediation of *Paulownia fortunei* (Seem) Hemsl. Ecotoxicology dpoi:. doi:10.1007/s10646-011-0836-5

Kim S, Rodriguez M, Suh J, Song J (2003) Emergent effects of heavy metal pollution at a population level: *Littorina brevicula* a study case. Mar Pollut Bull 46:74–80

Klaper R, Rees CH, Drevnick P, Weber D, Sandheinrich M, Carvan M (2006) Gene expression changes related to endocrine function and decline in reproduction in fathead minnow (*Pimephales promelas*) after dietary methylmercury exposure. Environ Health Perspect 114:1337–1343

Lande R (1988) Genetics and demography in biological conservation. Science 241:455–1460

Laurinolli M, Bendell-Young L (1996) Copper, zinc, and cadmium concentrations in *Peromyscus maniculatus* sampled near an abandoned copper mine. Environ Contam Toxicol 30:481–486

Layne JN (1968) Ontogeny. In: King JA (ed) Biology of *Peromyscus* (Rodentia). The American Society of Mammalogists, Provo, Utah, pp 148–253, Publication number 2

Lefèbvre C, Vernet P (1990) Microevolutionary processes on contaminated deposits. In: Shaw J (ed) Heavy metal tolerance in plants: evolutionary aspects. CRC Press, Boca Raton, pp 285–300

Levengood J, Heske E (2008) Heavy metal exposure, reproductive activity, and demographic patterns in white-footed mice (*Peromyscus leucopus*) inhabiting a contaminated wetland. Sci Total Environ 389:320–328

Lynch M, Conery J, Burger R (1995) Mutation accumulation and the extinction of small populations. Am Nat 146:489–518

Ma W, Denneman W, Faber J (1991) Hazardous exposure of ground-living small mammals to cadmium and lead in contaminated terrestrial ecosystems. Arch Environ Contam Toxicol 20:266–270

Maes GE, Raeymaekers JAM, Pampoulie C, Seynaeve A, Goemans G, Belpaire C, Volckaert FAM (2005) The catadromous European eel *Anguilla anguilla* (L.) as a model for freshwater evolutionary ecotoxicology: relationship between heavy metal bioaccumulation, condition and genetic variability. Aquat Toxicol 73:99–114

Mares MA, Ernest KA (1995) Population and community ecology of small mammals in a gallery forest of central Brazil. J Mammal 76:750–768

Matson C, Lambert M, McDonald T, Autenrieth R, Donnelly K, Islamzadeh A, Politov D, Bickham J (2006) Evolutionary toxicology: population-level effects of chronic contaminant exposure on the marsh frogs (*Rana ridibunda*) of Azerbaijan. Environ Health Persp 114:547–552

Medina M, Correa J, Barata C (2007) Micro-evolution due to pollution: possible consequences for ecosystem responses to toxic stress. Chemosphere 67:2105–2114

Miller MP (2000) Tools for populations genetic analyses (TFPGA) 1.3: A window program for the analyses of allozyme and molecular population genetic data computer software distributed by author.

Mills SL, Allendorf FW (1996) The one-migrant-per-generation rule in conservation and management. Coserv Biol 10:150–158

Morales EO, Carrillo FC (2010) Plan municipal de desarrollo de Tlaquiltenango, Morelos. H. Ayuntamiento de Tlaquiltenango, Morelos. http://www.tlaquiltenango.gob.mx/Transparencia/Obligaciones_Contable_Administrativas/OCA15_Plan_desarrollo_estatal%20doc.pdf. Accessed 19 September 2012

Morgan AJ, Kille P, Sturzenbaum SR (2007) Microevolution and ecotoxicology of metals in invertebrates. Environ Sci Technol 41:1085–1096

Mossman CA, Waser PM (2001) Effects of habitat fragmentation on population genetic structure in the white-footed mouse (*Peromyscus leucopus*). Can J Zool 79:285–295

Mullen L, Hirschmann R, Prince K, Glenn T, Dewey M, Hoekstra H (2006) Sixty polymorphic microsatellite markers for the old field mouse developed in *Peromyscus polionotus* and *Peromyscus maniculatus*. Mol Ecol Notes 6:36–40

Mussali-Galante P (2008) Estudio sobre la inducción de daño al ADN en sangre periférica de individuos expuestos a metales en al agua de bebida, en la población de Huautla, Morelos. Dissertation, Universidad Nacional Autónoma de México

Nacci D, Hoffman GR (2008) Genetic variation in population-level ecological risk assessment. In: Barnthouse LW, Munns WR, Sorensen MT Jr (eds) Population-level ecological risk assessment. Taylor and Francis, New York, pp 93–112

Nei M (1978) Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 89:583–590

Page RDM (1996) TreeView: an application to display phylogenetic trees on personal computers. Comput Appl Biosci 12:357–358

Pascoe GA, Blanchet RJ, Linder G, Lande R (1994) Bioavailability of metals and arsenic to small mammals at a mining waste-contaminated wetland. Genetics and demography in biological conservation. Science 241:455–1460

Peakall DB (1992) Animal biomarkers as pollution indicators, Ecotoxicological Series No.1. Chapman & Hall, London

Peakall R, Lindenmayer D (2006) Genetic insights into population recovery following experimental perturbation in a fragmented landscape. Biol Conserv 132:520–532

Peakall R, Ruibal M, Lindmeyer D (2003) Spatial correlation analysis offers new insights into gene flow in the Australian bush rat, *Rattus fuscipes*. Evolution 57:118–295

Phelps KL, McBee K (2009) Ecological characteristics of small mammal communities at a superfund site. Amer Midl Nat 161:57–68

Pra D, Rech-Frenke SI, Giulian R, Yoneama ML, Ferraz-Diaz J, Erdtmann B, Pegas-Henriques JA (2008) Genotoxicity and mutagenicity of iron and copper in mice. Biometals 21:289–297

Queller DC, Goodnight KF (1989) Estimating relatedness using genetic markers. Evolution 43:258–275

Ricketts HJ, Morgan AJ, Spurgeon DJ, Kille P (2004) Measurement of annetocin gene expression: a new reproductive biomarker in earthworm ecotoxicology. Ecotox Environ Safety 57:4–10

Rogstad S, Keane B, Collier M (2003) Minisatellite DNA mutation rate in dandelions increases with leaf-tissue concentrations of Cr, Fe, Mn, and Ni. Environ Toxicol Chem 22:2093–2099

Rzedowski J (2006) Vegetación de México. Fondo de Cultura Económica, Mexico

Sánchez CH, Romero MLA (1992) Mastofauna silvestre del ejido el Limón, municipio de Tepalcingo, Morelos. Univ Cienc Tec 2:87–95

Scheirs J, Coan A, Covaci A, Beernaert J, Kayawe M, Caturla M, Wolf H, Baert P, Van Oostveldt P, Verhagen R, Blust R, Coen W (2006) Genotoxicity in wood mice (*Apodemus sylvaticus*) along a pollution gradient: exposure age and gender-related effects. Environ Toxicol Chem 25:2154–2162

Secretaría de Economía (2011) Panorama minero del Estado de Morelos. Servicio Geológico Mexicano, serie panorama minero de los estados, Mexico

Sheffield SR, Sawicka-Kapusta K, Cohen JB, Rattner BA (2001) Rodentia and Lagomorpha. In: Shore RF, Rattner BA (eds) Ecotoxicology of wild mammals. John Wiley and Sons, New York, pp 215–314

Shore RF, Douben PE (1994) Predicting ecotoxicological impacts of environmental contaminants on terrestrial small mammals. Rev Environ Contam T 134:49–89

Shugart L, Theodorakis C (1998) New trends in biological monitoring: application of biomarkers to genetic ecotoxicology. Biotherapy 11:119–127

Smith PN, Cobba GP, Harper FM, Adair BM, McMurry ST (2002) Comparison of white-footed mice and rice rats as biomonitors of polychlorinated biphenyl and metal contamination. Environ Pollut 119:261–268

Sneath PHA, Sokal RR (1973) Numerical taxonomy. The principles and practice of numerical classification. WH Freeman, San Francisco, 573 p

Sommer S (2003) Effects of habitat fragmentation and changes of dispersal behavior after a recent population decline on the genetic variability of noncoding and coding DNA of a monogamous Malagasy rodent. Mol Ecol 12:2845–2851

Staton J, Schizas N, Chandler G, Coull B, Quattro J (2001) Ecotoxicology and population genetics: the emergence of "phylogeographic and evolutionary ecotoxicology". Ecotoxicology 10:217–222

Statsoft (2000) Statistica for Windows, v. 5.1. Computer program manual. Tulsa, StatSoft Inc

Stockley P, Searle JB, Macdonald DW, Jones CS (1993) Female multiple mating behavior in the common shrew as a strategy to reduce inbreeding. Proc R Soc Lond B 254:173–179

Swofford DL, Olsen GJ (1990) Phylogeny reconstruction. In: Moritz C, Hillis DM (eds) Molecular systematics. Sinauer Associated Inc, Massachusetts, pp 411–501

Theodorakis C (2001) Integration of genotoxic and population genetic endpoints in biomonitoring and risk assessment. Ecotoxicology 10:245–256

Tovar-Sánchez E, Cervantes LT, Martínez C, Rojas E, Valverde M, Ortiz-Hernández ML, Mussali-Galante P (2012) Comparison of two wild rodent species as sentinels of environmental contamination by mine tailings. Environ Sci Pollut Res 19:1677–1686

Ungherese G, Mengoni A, Somigli S, Baroni D, Focardi S, Ugolini A (2010) Relationship between heavy metals pollution and genetic diversity in Mediterranean population of the sandhopper *Talitrus saltator* (Montagu) (Crustaceae, Amphipoda). Environ Pollut 158:1638–1643

Valavanidis A, Vlachogianni T (2010) Metal pollution in ecosystems: ecotoxicology studies and risk assessment in the marine environment. Sci Adv Environ Toxicol Ecotox Issues. www.chem-tox-ecotox.org

Van de Zande L, Van Apeldoorn RC, Blijdenstein AF, De Jong D, Van Delden W, Bijlsma R (2000) Microsatellite analysis of population structure and genetic differentiation within and between population of the root vole, *Microtus oeconomus* in the Netherlands. Mol Ecol 9:1651–1656

Van Straalen N (1999) Genetic biodiversity in toxicant-stressed populations. Prog Environ Sci 1:195–201

Van Straalen N, Timmermans M (2002) Genetic variation in toxicant-stressed populations: an evaluation of the "genetic erosion" hypothesis. Hum Ecol Risk Assess 8:983–1002

Vargas V (2010) Estructura genética del roedor *Baiomys musculus* (Muridae) en la selva seca caducifolia en el estado de Morelos. Dissertation. Universidad Autónoma del Estado de Morelos

Volke ST, Velasco TA, De la Rosa PA, Solórzano OG (2004) Evaluación de tecnologías de remediación para suelos contaminados con metales. Etapa I. Secretaría de Medio Ambiente y Recursos Naturales, Mexico

Volke ST, Velasco TA, De la Rosa PA, Solórzano OG (2005) Evaluación de tecnologías de remediación para suelos contaminados con metales. Etapa II. Secretaría de Medio Ambiente y Recursos Naturales, Mexico

Vucetich LM, Vucetich JA, Cleckner LB, Gorski PR, Peterson RO (2001) Mercury concentrations in deer mouse (*Peromyscus maniculatus*) tissues from Isle Royale National Park. Environ Pollut 114:11–38

Weber JN, Peters M, Tsyusko O, Linnen C, Hagen C, Schable N, Tuberville T, Mckee A, Lance S, Jones K, Fisher H, Dewey M, Hoekstra H, Glenn C (2010) Five hundred microsatellite loci for *Peromyscus*. Conserv Genet 11:1243–1246

Weir BS (1996) Genetic data analysis II: methods for discrete population genetic data. Sinauer Associated Inc, Massachusetts

Werre F, Ortiz-Hernández L (2000) Monografía geologica-minera del estado de Morelos. Consejo de Recursos Minerales, Mexico

WHO World Health Organization (2007) Health risks of heavy metals from long range transboundary air pollution. WHO Regional Office for Europe, Copenhagen

Yap CK, Tan SG, Ismail A, Omar H (2004) Allozyme polymorphisms and heavy metal levels in the green-lipped mussel *Perna viridis* (Linnaeus) collected from contaminated and uncontaminated sites in Malaysia. Environ Inter 30:39–46

Yap CK, Chua BH, Teh CH, Tan SG, Ismail A (2007) Primers of RAPD markers and heavy metal concentrations in *Perna viridis* (L.), collected from metal-contaminated and uncontaminated coastal waters: are they correlated with each other? Russian J Genet 43:544–550

Yap CK, Chong CM, Tan SG (2011) Allozyme polymorphism in the horseshoe crabs *Carcinoscorpius rotundicauda* collected from polluted intertidal area in Peninsular Malaysia. Environ Monit Assess 174:389–400

Yeh FC, Yang R, Boyle T (1999) PopGene Ver. 1.31. University of Alberta, Canada

Zar J (2010) Biostatistical analysis. Prentice-Hall, New-Jersey

APÉNDICE B

Rev. Int. Contam. Ambie. 29 (1) 117-140, 2013

REVIEW

BIOMARKERS OF EXPOSURE FOR ASSESSING ENVIRONMENTAL METAL POLLUTION: FROM MOLECULES TO ECOSYSTEMS

Patricia MUSSALI-GALANTE[1*], Efraín TOVAR-SÁNCHEZ[2], Mahara VALVERDE[1] and Emilio ROJAS DEL CASTILLO[1]

[1] Departamento de Medicina Genómica y Toxicología Ambiental, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México
[2] Departamento de Sistemática y Evolución, Centro de Investigación en Biodiversidad y Conservación, Universidad Autónoma del Estado de Morelos, Av. Universidad 1001, Col. Chamilpa, Cuernavaca, Morelos. CP. 62209, México
*Autora responsable: pmussali@yahoo.com.mx

(Recibido marzo 2012, aceptado enero 2013)

Key words: metals, biomarkers, ecological markers, genetic toxicology, ecotoxicology, "omic" technologies

ABSTRACT

Metals are among the most prevalent substances released into the environment that have a profound effect on living organisms. Chronic environmental exposures usually exert a continuum of biological responses across levels of biological organization, ranging from alterations in molecules, compromising individual health and putting ecosystem integrity at risk. Such scenarios have triggered the research to establish "early-warning" signals, or "biomarkers", reflecting the adverse biological responses towards environmental pollution. In this review, we assess the different types of biomarkers most used to analyze environmental metal pollution across all levels of biological organization and in each section representative examples in human and animal species and/or wild populations are given. Also, the "omics" approach is described and how these novel technologies are reinventing the field of toxicology, providing "molecular signatures" of exposure, enabling a more robust risk assessment than has ever been achieved previously. Finally, conclusions and suggestions are given, highlighting why future efforts must focus on integrating biomarker response across levels of biological organization, which integrate realistic exposures using multi-species and multiple-biomarkers with prognostic value to resolve or at least have a closer insight into complex environmental problems.

Palabras clave: metales, biomarcadores, marcadores ecológicos, genética toxicológica, ecotoxicología, tecnología genómica

RESUMEN

Los metales se incluyen dentro de las substancias más persistentes emitidas al ambiente, los cuales tiene efectos importantes sobre los seres vivos. La exposición ambiental crónica a los metales generalmente resulta en un continuo de respuestas biológicas que se da en todos los niveles de organización biológica. Estas respuestas pueden observarse desde alteraciones a nivel molecular, comprometiendo la salud del individuo, hasta poner en riesgo la salud del ecosistema. Lo anterior ha impulsado la

investigación científica para establecer "señales tempranas de alerta" mediante el uso de "biomarcadores", los cuales reflejen los efectos biológicos adversos producidos por los contaminantes ambientales. En este trabajo se revisan los biomarcadores más utilizados para estudiar la contaminación ambiental producida por metales, en todos los niveles de organización biológica y en cada sección se dan ejemplos representativos en humanos, especies animales y poblaciones silvestres. Además, se describe desde la perspectiva de las ciencias ómicas, como estas metodologías han reinventado el campo de la toxicología, proporcionando "huellas moleculares" de exposición, permitiendo así un análisis de riesgo más robusto el cual no se había alcanzado antes. Finalmente, se dan conclusiones y sugerencias resaltando la razones de por qué los esfuerzos futuros deben enfocarse en la integración de las respuestas proporcionadas por los biomarcadores en todos los niveles de organización biológica, que consideren exposiciones más apegadas a la realidad, mediante diseños experimentales más rigurosos utilizando multiespecies y multibiomarcadores con valor predictivo para resolver, o entender mejor los problemas ambientales complejos.

INTRODUCTION

The environment is continuously loaded with foreign chemical substances, released by anthropogenic activities. As a result, many wildlife and human populations are exposed to a variety of chemical agents which may lead to a collection of biological effects. Among environmental pollutants, metals have been identified among the most toxic elements to nearly all living organisms (EPA 2000). The relationship between metal toxicity and a plethora of effects is well established. Studies from populations exposed to metals, were among the first to establish quantitative relationships between the external exposure, the internal dose, and the early effects (Bernard 2008).

Organisms integrate exposure to contaminants in their environment and respond in some measurable and predictable way, being these responses observed and measurable across different levels of biological organization (Bickham *et al.* 2000). In the field of toxicology, it is essential to be able to measure the exposure to a toxic agent, the extent of any toxic response and also to predict the likely effects. Hence, integrating measures of different types of responses to toxic stress of exposed individuals and populations, offers a powerful tool for documenting the extent of exposure and the effects of environmental metal contamination. Tools that enable this to be done are called "biological markers" or "biomarkers". For these reasons, the use of biomarkers for environmental monitoring of individuals and populations exposed to chemical pollution has gained much attention in the last decades, because it offers great opportunities for a fast and sensitive detection of chemical stresses within organisms (Peakall and Shugart 1992, Handy *et al.* 2003).

The use of biomarkers in environmental health was described in a series of publications issued by the Board of Environmental Studies in Toxicology of the National Research Council (NRC 1987, 1989) of the USA. The NRC defines biomarkers as "Indicators of events in biological systems or samples" and was further described as "tools that can be used to clarify the relationship, if any, between exposure to a xenobiotic substance and disease". Also, the NRC classified biomarkers into three categories based on their relation to the exposure-disease continuum: biomarkers of exposure, effect and susceptibility. Some years later, Lagadic *et al.* (1994) referred to biomarkers as "biochemical sub-lethal changes resulting from individual exposure to xenobiotics". These definitions denote that many researches focus on biomarkers as measures at the cellular or sub-cellular levels, as in the case of molecular epidemiology and genetic toxicology, where measurements of toxic responses are routinely used to infer cause-effect relationships between biomarker response and health effects of the exposed individuals (Perera 2000). Also, the former definitions restrict the term biomarker to measurements at or below the level of individuals. Hence, it becomes important to consider that there are other types of biomarkers that attempt to measure effects of chemical pollution at the population, community and even at the ecosystem level (ecotoxicology). This reflects the fact that pollutants can exert their influence at all levels of biological organization (Lagadic *et al.* 1994, Peakall 1994). In this context, Handy *et al.* (2003) expanded the concept as "the identification of specific molecular, biochemical, physiological and behavioral changes in populations following pollutant exposure". Both approaches try to reveal cause-effect relationships between the initial exposure and the subsequent effects, based on the use

of biomarkers, but in different levels of biological organization.

In this review, we assess the most common biomarkers used in each level of biological organization. The first section, deals with biological responses exerted by metals from molecules to individuals. The next section, addresses biological responses from populations to ecosystems. In each section, representative examples concerning environmental metal exposures in humans and animal species (individuals and populations) are given, in order to illustrate how the use of biomarkers is suitable for studying metal exposures.

Also, a new approach is described, the "omics" approach, where the search for new biomarkers becomes possible. These novel technologies offer added value compared with classical testing with whole organisms because they provide information concerning the molecular basis of exposure "molecular signatures" and act as "early warning" signals, enabling a more robust environmental monitoring than has ever been achieved previously (Snape *et al.* 2004).

DEFINITIONS AND TYPES OF BIOMARKERS: FROM MOLECULES TO INDIVIDUALS

Many metals are essential to living organisms but some of them are highly toxic or become toxic at high concentrations, these include iron (Fe), Copper (Cu), Zinc (Zn), Cobalt (Co), Molybdenum (Mo), and Manganese (Mn). Light metals such as Sodium (Na), Potassium (K), and Calcium (Ca) play important biological roles. Metals such as Mercury (Hg), Lead (Pb), Niquel (Ni), Chromium (Cr), Cadmium (Cd), and Arsenic (As) are generally not required for metabolic activity and are toxic to living organisms at quite low concentrations (Valavanidis and Vlachogianni 2010). Other metals such as Vanadium (V) which is present in almost all-living organisms but its essentiality in cellular functions is yet to be established, is also capable of inducing toxic effects in various species (Rodríguez-Mercado and Altamirano-Lozano 2006). As a consequence, the toxicological effects of metals have been widely studied, where it has been recognized that the relationship between exposure and disease is as a multistage process which includes external exposure, internal dose, early biological effects, altered structure and function and finally clinical changes or disease (Link *et al.* 1995, Vanden-Heuvel and Davis 1999).

When characterizing toxicological responses, it is desirable to distinguish each step in this continuum. Biomarkers signify these alterations in biological systems and may be indicators of exposure, effect or susceptibility and may overlap sometimes (Perera 1996, Perera and Weinstein 2000, Jakubowski and Trzcinka-Ochoka 2005, Nordberg 2010).

Biomarkers of exposure: "An exogenous substance or its metabolite or the product of an interaction between a xenobiotic agent and some target molecule or cell that it is measured in a compartment within an organism". These types of biomarkers are also known as "biological dosimeters" or biomarkers of internal dose, and when they measure the product of the interaction with target molecules they are regarded as "biomarkers of biological effective dose" (Timbrell 1998).

Biomarkers of effect: "A measurable biochemical, genetic, physiological, behavioral or other alteration within an organism that, depending on the magnitude, can be recognized as associated with an established or early health impairment or disease" (Timbrell 1998).

Biomarkers of susceptibility: "An indicator of an inherent or acquired ability of an organism to respond to the challenge of exposure to a specific xenobiotic substance" (Pavanello and Clonfero 2000, Sakai 2000).

TYPES OF BIOMARKERS OF EXPOSURE

Biomarkers of internal dose: these are the most used, because of their precision, reliability and relevance to individual risk (Perera and Weinstein 2000, Aitio *et al.* 2007, Nordberg 2010). They have been used in combination with measures of external exposure. Currently, highly sensitive analytical methodologies make possible to measure very low concentrations of a chemical substance or its metabolite in various cell types, organs or body fluids. These types of biomarkers take into account individual differences in absorption, metabolism, bioaccumulation and excretion of the compound in question and indicate the actual dose of the substance within an organism and in specific tissues (Perera and Weinstein 2000).

Examples of internal dosimeters of metal exposure include: hair, nail, blood, and urinary levels of total inorganic As or its metabolites (Hughes 2006, Fowler *et al.* 2007), Pb blood concentrations (Bjorkman *et al.* 2000, Aitio *et al.* 2007), Cd blood, urine and kid-

ney concentrations (Clarkson *et al.* 1988, Nordberg *et al.* 2007, Nordberg 2010) and methylmercury in hair (Jakubowski and Trzcinka-Ochocka 2005) and V concentrations in kidney and liver (Gummow *et al.* 2006). For a more detailed review and examples see Fowler (1987, 1992).

Although biomarkers of internal dose are a valuable tool for assessing chemical exposures, they do not indicate the extent to which a given compound has interacted with molecular and cellular targets. For this reason assays have been developed to measure the "biological effective dose" (Perera and Weinstein 2000).

Biomarkers of biological effective dose: These types of biomarkers occur early in the exposure-to-disease pathway. Some of them have been shown to be associated with increased risk of developing diseases such as cancer. As a result, they are considered as important tools for investigating mechanisms behind exposure-induced adverse health effects (Perera 2000, Sorensen *et al.* 2003). The best known examples are DNA-adducts.

The study of DNA-adducts is motivated by the fact that many environmental contaminants and some metals are thought to exert their genotoxic effects through covalent binding with DNA (Perera and Weinstein *et al.* 2000, Poirier 2004, Gallo *et al.* 2008). DNA-adducts are addition products formed by covalent binding of all or part of a metal molecule to chemical moieties in DNA; adducts are formed when an activated chemical species (electrophilic, positively charged metabolite) binds covalently to negatively charged moieties. In other words, they represent the amount of a given metal that has reacted with critical cellular macromolecules such as DNA or proteins in a given tissue (Ehrenberg *et al.* 1996). In this context, DNA-adducts are among the most informative biomarkers of exposure to genotoxic agents (Poirier 2004).

The quantification of DNA-adducts gives information about the biologically effective dose of a metal reaching the DNA in cells. As a result, they represent the amount of the metal that has been absorbed by the body, undergone metabolic activation, become bound to cellular DNA and has not been repaired (Rundle *et al.* 2002, Gallo *et al.* 2008). DNA-adducts if not repaired or repaired inadequately, may lead to mutation and alteration of gene function (Farmer 2004, Jakubowski and Trzcinka-Ochocka 2005, Swenberg *et al.* 2008).

Early studies utilized column chromatography to examine adduct formation, but this technique has a detection limit of 1 adduct per 10^6 nucleotides (Swenberg *et al.* 2008). Thereafter, Randerath *et al.* (1994) developed one of the most used techniques to analyze the extent of DNA-adducts, which is ^{32}P-postlabelling, detecting at least one adduct per 10^8 nucleotides. Recently, using accelerator mass spectrometry, 1 adduct per 10^{12} bases are possible to detect, which is probably 1 adduct per cell (Singh and Farmer 2006, Swenberg *et al.* 2008). It is important to mention that although the formation of DNA-adducts is not the main mechanism of toxicity of metals, many authors mention that they may form DNA-adducts directly, as in the case of Cr (Singh *et al.* 1998, Zhitkovich 2005) and water soluble Ni compounds (Muller *et al.* 1999), and indirectly (through formation of free radicals and reactive oxygen species (ROS)) as in the case of As (Wang *et al.* 2001, Bau *et al.* 2002, Rossman 2003, Méndez-Gómez *et al.* 2008).

TYPES OF BIOMARKERS OF EFFECT

Biomarkers of effect are perhaps best regarded as indicators of early changes that could later lead to clinical disease (Mutti 1995). There are situations where biomarkers of exposure are not sufficient to predict potential adverse effects. In such situations, biomarkers of effect are used to understand if a change in their distribution has occurred as a result to the chemical exposure. Hence, biomarkers of effect are not proof of disease caused by environmental pollution but tools to understand a process that might eventually lead to adverse effects (Watson and Mutti 2004). Biomarkers of effect give measures of the alterations on important genetic targets like DNA, causing DNA-breaks, chromosome aberrations and micronucleus. Biomarkers of biochemical effect provide information about oxidative damage in DNA and proteins, alterations in a wide range of enzymes like DNA-repair enzymes, and metal-binding proteins, among others (Frenzilli *et al.* 2009, Rojas 2009).

DNA single (SSB) and double strand breaks (DSB): Another approach for evaluating the possible consequences of environmental metal pollution involves the assessment of genotoxic damage measured as DNA-breaks. Most metals interact indirectly with DNA, via generation of ROS, causing single and double strand breaks (Valko *et al.* 2006, Mussali-Galante *et al.* 2007, Frenzilli *et al.* 2009).

The DNA molecule must undergo continuous maintenance to sustain its integrity. Several key mechanisms in DNA-repair processes involve the

degradation of a short stretch of DNA leading to a transitory break in a single DNA strand. The incidence of such strand breaks may be enhanced both as a direct result of metal exposure or as an indirect effect of repair processes (Shugart and Theodorakis 1994, Hebert and Murdoch 1996). Measurement of DNA-breaks induced by metal exposure is common there are several approaches to quantify the frequency of DNA strand breakage: the alkaline unwinding assay (Shugart 1988), the single cell gel electrophoresis assay or "comet assay" (Rojas *et al.* 1999), chromosomal aberrations (Obe *et al.* 2002), alkaline elution (Koch and Giandomenico 1994) and sister chromatid exchanges (Perry and Wolf 1974), among others (Ahnstrom 1988, Lindberg *et al.* 2007).

The comet assay, is a rapid, simple, and sensitive technique for analyzing DNA breakage of single and double strands, depending of pH conditions, in individual cells (Singh *et al.* 1988, Silva *et al.* 2000, Tice *et al.* 2000, Mussali-Galante *et al.* 2005, Rojas 2009). In principle, any organism is suitable for the comet assay and small cell samples are needed. As a result, the comet assay has become one of the major tools for environmental biomonitoring studies (Valverde and Rojas 2009). It is important to mention that even when the Comet assay is sensitive to detect strand breaks, it is a nonspecific chemical biomarker of genotoxicity (Dhawan *et al.* 2009). However, the versatility in terms of cell types used to determine DNA-breaks as a consequence of metal exposure is illustrated in the following examples: Humans exposed to As (Abernathy *et al.* 1999, Calderón *et al.* 2003, Pandey *et al.* 2007), humans exposed to V (Ehrlich *et al.* 2008), coelomocytes exposed to Ni (Reinecke and Reinecke 2004), earthworms exposed to Cd (Fourie *et al.* 2007), grasshoppers exposed to Zn (Augustyniak *et al.* 2006), birds (Baos *et al.* 2006, Pastor *et al.* 2001, 2004), mussels (Machella *et al.* 2006) and wild mice species exposed to heavy metal mixtures (Leon *et al.* 2007, Tovar-Sánchez *et al.* 2012). More detailed examples are given by Dhawan *et al.* (2009) and Frenzilli *et al.* (2009).

Chromosome aberrations (CA): From the vast scientific literature assessing CA as a biomarker of effect, it is evident that cytogenetic biomarkers have been a valuable tool for studying the most important environmental hazards occurring in the past decades. The use of valid biomarkers of risk in populations exposed to genotoxic agents is the most suitable and well-established approach for analyzing many modern exposures (Tucker and Preston 1996, Bonassi *et al.* 2005). CA are induced by agents that damage chromosomal DNA (Natarajan 1976). A large amount of evidence demonstrates that DNA-DSB are the principal lesions in the process of CA formation (Pfeiffer *et al.* 2000). DSB arise spontaneously at high frequencies through a variety of cellular processes (Natarajan 1976, Bonassi *et al.* 2005). However, the majority of chemical mutagens are not able to induce DSB directly but lead to other lesions in chromosomal DNA which, during repair or DNA synthesis, may give rise to DSB and eventually to CA (Tucker and Preston 1996, Obe *et al.* 2002).

Many studies assessing the frequency of CA and other genotoxic endpoints resulting for environmental metal exposure have been conducted in As exposed human populations (Ostrosky-Wegman *et al.* 1991, Gonsebatt *et al.* 1997) and in animal species inhabiting superfund sites. In this context, the studies of McBee *et al.* (1987) and McBee and Bickham (1988) where the first to report higher levels of karyological damage in two wild rodent species living in a metal contaminated site. Also in many fish species (Prein *et al.* 1978, Hooftman and Vink 1981) in orthopterans (*Tetrix tenuicornis)* living in zinc-lead mine spoils (Warchałowska-Śliwa *et al.* 2005), and in dipterans (*Chironomus riparius*) inhabiting a polluted site (Zn, Cd, Pb, Cu) (Michailova *et al.* 1996).

It is important to mention that unlike chemical DNA-adducts, chromosomal aberrations are a non-chemical specific biomarker (Perera 2000).

Micronuclei (MN): As their name suggests, micronuclei are masses of DNA (resembling small nuclei) found in the cytoplasm, rather than being contained within the nuclear membrane. Micronuclei form when acentric or centromeric chromosome fragments are unable to attach to a spindle fiber during cell division or when an intact chromosome is excluded from the nucleus because of defective cell division. Hence, micronuclei may be a consequence of either chromosomal breakage or dysfunction of the spindle mechanism (Lindberg *et al.* 2007). These types of micronuclei can be distinguished (Boei and Natarajan 1995), and there is evidence that genotoxic agents can be differentiated by whether they induce chromosomal breakage or loss (Chen *et al.* 1994, Fenech and Crott 2002) and/or centromeric modifications (Fenech *et al.* 1999). They have been studied for many years, in experimental research as well as in environmental monitoring. In the last decade, MN assay has gained a lot of attention because it offers several advantages: a) MN can be observed in almost any eukaryotic cell type, b) Speed, ease and low cost of the analysis, and c) the non-requirement for me-

taphase cells. Thus, MN analyses can be employed in studies with different experimental conditions, in a wide variety of animal species (Bonassi *et al.* 2005). For many years, research employing the MN assay in environmental exposures has been conducted in individuals exposed to As in drinking water (Fenech *et al.* 1999, Basu *et al.* 2004).

Recently, research has been carried out to evaluate the clastogenic and/or aneugenic activity of different environmental metal pollutants in natural animal populations (Bolognesi and Hayashi 2011). For this purpose, the fish erythrocyte micronucleus test has been used as an informative biomarker to evaluate the clastogenic potential of metals in water (Al-Sabti 1994, Minissi *et al.* 1996, Russo *et al.* 2004). Other examples that report statistically high frequencies of MN include, eels (*Anguilla anguilla*) exposed to Cd and Hg (Sánchez-Galán *et al.* 2001), and the wood mouse (*Apodemus silvaticus*) exposed to Cd, Fe, Zn, Cu, Mn, Mo and Cr (Sánchez-Chardi *et al.* 2007).

Sister chromatid exchange (SCE): This assay is a well-known cytogenetic technique that has been used extensively to assess DNA damage at the chromosomal level (Hagmar *et al.* 1994). SCE occur as a normal feature of cell division in mammalian cells. They are believed to represent the interchange of DNA replication products at apparently homologous loci which involve DNA breakage and reunion (Gauthier *et al.* 1999, Wilson and Thompson 2007). During the S-phase of the cell cycle, DNA is replicated, and each chromosome becomes duplicated into two closely associated daughter chromatids that are linked tightly at the centromere. Sister chromatids are visible cytologically in late prophase and early metaphase of mitosis before chromosome segregation occurs (Latt 1973, Kaina 2004). Hence, SCE is the process whereby the sister chromatids effectively break and rejoin with one another, physically exchanging regions (Kato 1974, Perry and Wolf 1974, Wilson and Thompson 2007). While SCE are readily observed experimentally, the mechanisms that mediate SCE are not fully understood and controversial results have been reported (Ohno *et al.* 1982, Hartmann and Speit 1994, Fogu *et al.* 2000, Wilson and Thompson 2007, Tapisso *et al.* 2009). Particular types of genotoxic chemicals like bifunctional alkylating agents are in general potent inducers of SCE, presumably because homologous recombination is required to repair the resulting broken replication forks that arise during crosslink releasing (Thompson 2005). Metals that are known to induce SCE are cadmium, chromium, aluminum, arsenic, lead, vanadium and zinc (Sivikova and Dianovsky 1995, Bilban 1998, Mouron *et al.* 2004). This evidence comes mainly from *in vitro* (Fan *et al.* 1996, Basu *et al.* 2001, Rodriguez-Mercado *et al.* 2003, Mouron *et al.* 2004) and *in vivo* (Mukherjee *et al.* 1988, Gennart *et al.* 1993, Lai *et al.* 1998, Tapisso *et al.* 2009) studies or from studies of humans exposed to arsenic in drinking water (Ostrosky *et al.* 1991, Lerda 1994, Basu *et al.* 2001, Rossman 2003). However, there are very few studies assessing the induction of SCE in wild animal populations [Arctic beluga whale (*Delphinapterus leucas*), Gauthier *et al.* 1999] exposed to environmental metal stress in comparison with other biomarkers of early effect. Since these biomarkers (SSB, DSB, MN, CA) analyze different types of DNA damage, which can have dissimilar sensitivities to metals, these assays should be used complementary, along with the inclusion of SCE for biomonitoring exposure to genotoxic compounds in the natural habitat of different animal populations.

BIOMARKERS OF BIOCHEMICAL EFFECT

Oxidative damage: Under normal physiological conditions in all aerobic organisms, there is a balance maintained between endogenous oxidants and numerous enzymatic and non-enzymatic antioxidant defenses (Halliwell and Gutteridge 1999). When an imbalance occurs, oxidants produce extensive oxidative damage to macromolecules such as DNA, proteins and lipids, which, in turn, contributes to aging, cancer, and other degenerative diseases.

Nearly 100 different oxidative DNA modifications have been identified, ranging from modified bases to DNA-breaks in a wide variety of animals and human cells exposed to chemical agents (Dizdaroglu 1992, Cadet *et al.* 2002). In all cells, altered DNA is repaired enzymatically, while misrepaired DNA can result in mutations leading to genomic instability and cancer (Kawanishi *et al.* 2001). Although a broad range of DNA alterations are produced during oxidative damage to DNA, most interest has focused on guanine oxidation products, among them are, 8-hydroxyguanine (8-oxo-G), 8- hydroxyguanosine (8-oxyGuo) and 8-hydroxy-2-deoxyguanosine (8-OHdG). One of the most abundant lesions is 8-OHdG, which is formed *in vivo* and can be measured quantitatively in cells following hydrolysis of the DNA to component bases (Valavanidis *et al.* 2009). This lesion is a major product of hydroxyl radical attack on DNA and of maximum biological importance. Also, 8-OHdG has attracted particular attention because it causes

G-to-T transversions and its presence may lead to mutagenesis (Hayes 1997, Wong *et al.* 2005, Valavanidis *et al.* 2009). Measurements of 8-OHdG, or its corresponding nucleoside, after repair processes results in the excised 8-OHdG adduct being excreted in urine, and because of its easy collection, these biomarkers are among the most widely used markers of oxidative DNA damage (Wong *et al.* 2005). The 8-OHdG level in DNA isolated from tissue is believed to exemplify the steady state damage of DNA being a result of damage and repair, while 8-OHdG excreted in urine is alleged to be an indicator of total DNA excision repair within an organism. As it is assumed that DNA repair under normal conditions is almost complete, 8-OHdG excretion is also a marker of the rate of total DNA damage (Loft and Poulsen 1999, Sorensen *et al.* 2003).

Because of its capacity to lose electrons, a metal is primarily thought to be toxic by virtue of its generation of ROS. Thus, exposure to high concentrations of a single heavy metal might result in its accumulation and potentially, oxidative damage (Limón-Pacheco and Gonsebatt 2009). Metals such as Fe, Mn, Ni, Cu, Cr, and V can generate ROS in biological systems causing oxidative damage in DNA and proteins (De Flora and Wetterhahn 1989, Gurgueira *et al.* 2002, Valavanidis *et al.* 2005, 2009, Valko *et al.* 2005). Specifically, the induction of 8-OHdG has been reported after *in vivo* exposure to As (Rossman 2003), Cd (Filipic and Hei 2004), Co (II) (Mao *et al.* 1996), Cr (VI) (Kuo *et al.* 2003), and V (Shi *et al.* 1996, Rodríguez-Mercado *et al.* 2003).

DNA repair enzymes: A general mechanism of carcinogenicity of As, Cd, Co, and Ni seems to be the inhibition of DNA repair enzymes and the consequent enhancement of DNA damage originally caused by other agents or raised spontaneously (Beyersmann 2002). Even though, the inhibition of DNA repair processes appears to be a common mechanism of action of some metal compounds, the steps affected seem to be rather different. One mechanism of repair inhibition is the displacement of essential metal ions such as Zn, Mn, Ni, and Co (Hartwig *et al.* 2002, Rossman 2003).

Some toxic metal ions have high affinities toward sulfhydryl (SH) groups, as a result, potential targets are the so called "zinc finger proteins". Although most zinc finger structures have been described as DNA-binding motifs in transcription factors, they have also been identified in several DNA repair enzymes (Rossman 2003). They include the mammalian XPA protein, the bacterial Fpg protein and the poly (ADP-ribose) polymerase.

Specifically, the Fpg protein is inhibited by Cd, Cu, and Hg and Ni and Co inhibit DNA binding of XPA (Asmuss *et al.* 2000). Also, poly (ADP-ribose) polymerase is inhibited by arsenite in mammalian cells (Hartwig *et al.* 2002, Schoen *et al.* 2004). For more comprehensive examples and molecular mechanisms see Hartwig *et al.* (1997) and Hartwig (1998, 2001).

These proteins have been used as biomarkers to analyze response to toxic metals. These findings have been observed at low concentrations, in most cases more than ten-fold below the cytotoxic level. Thus, under environmental exposure conditions, repair inhibition may contribute significantly to metal-induced toxicity and carcinogenicity (Méndez-Gómez *et al.* 2008). However, environmental exposures analyzing alterations in repair enzymes are scarce because the difficulty to link specific enzyme alterations exclusively to metal exposure.

Metallothioneins: For metals, much of the work in the area of biomarkers has focused on metallothioneins or metallothionein-like proteins (MT). These low-molecular weight, cysteine-rich metal-binding proteins are reported to play a key role in the binding and transport of various metals (Costa *et al.* 2008, 2009). The structure of these highly conserved proteins is linked to their role in the homeostasis of essential metals such as Zn and Cu and detoxification of toxic elements such as Cd and Hg. MT have several isoforms, apparently induced by different metals, the best known of which, MT-I and MT-II, are greatly induced by Cd and Zn (Viarengo *et al.* 1999, Romero-Isart and Vasak 2002).

MT induction is considered as a biochemical biomarker of exposure and of biologically effective dose, and can be used to point trace metal environmental exposures (Langston *et al.* 1998, Olsvik *et al.* 2001).

Another possible use of MT as a biomarker, involves the examination of the intracellular distribution of metals among cytosolic ligands, including MT. These types of changes offer several advantages as biomarkers; since molecular alterations are normally the first detectable, it becomes possible to quantify early responses to environmental metal stress. As a result, some authors have suggested that they may serve as markers of both exposure and effect (George and Olsson 1994, Olsvik *et al.* 2001). Hence, their use in environmental metal monitoring surveys has been well established (Perceval *et al.* 2004).

There is a considerable amount of literature concerning MT induction following metal envi-

ronmental exposure. Most of the studies have been conducted in humans and in aquatic animals. Some representative examples include: MT levels in liver and kidney of Canadian individuals exposed to Cd and Zn (Chung *et al.* 1986), MT induction in peripheral lymphocytes from Chinese individuals exposed to Cd (Lu *et al.* 2005), brown trout (*Salmo trutta*) exposed to Zn, Cd, Cu (Olsvik *et al.* 2001), the great tit (*Parus major*), along a metal pollution gradient (Pb, Cd) (Vanparys *et al.* 2008), and the fish (*Solea senegalensis*) exposed to As, Cd, Cr, Cu, Ni, Pb and Zn (Costa *et al.* 2009) and mussels (*Mytilus* sp.) exposed to V from an oil spill (Amiard *et al.* 2008). All these studies conclude that MT are modulated by heavy metals, being an informative and specific biomarker of chronic heavy metal exposure. More examples are reviewed in Petering and Fowler (1986), Nordberg (1998).

Aminolevulinic acid dehydratase (ALAD): It is well known that individuals exposed to lead may develop anemia, mainly from the interaction of lead with some enzymatic processes responsible for heme synthesis, like the inhibition of ALAD. ALAD is the second enzyme in the heme biosynthetic pathway which catalyses the condensation of two molecules of aminolevulinic acid to form one molecule of porphobilinogen. Erythrocyte ALAD activity is rapidly inhibited by lead exposure (Sakai *et al.* 1981). Therefore, determination of ALAD activity in erythrocytes is one of the most useful and well established biomakers for evaluating lead exposure, because the activity is extremely sensitive to and specific for blood lead concentration. If ALAD is inhibited, it is a clear indication of the presence of biological significant quantities of lead, but measurements of the activity of ALAD do not provide information on the presence of any other pollutants (Sakai *et al.* 1996, Sakai and Morita 1996, Sakai 2000). ALAD activity has been frequently measured in human adult individuals and children, as well as in animals after Pb environmental exposures were detected. For example, ALAD activity was significantly lower in a population of Indian children with the highest lead blood levels when compared to children with medium and low lead blood levels (Ahamed *et al.* 2005). Similar results were obtained among urban adolescents (Ahamed *et al.* 2006) and among adults and elderly people (Todd *et al.* 1996, Lee *et al.* 2006). In animals, ALAD activity has been frequently measured in birds (Johnson *et al.* 1999, Strom *et al.* 2002, Beyer *et al.* 2004, Vanparys *et al.* 2008), amphibians (Arrieta *et al.* 2004) and tortoises (Martinez *et al.* 2010).

BIOMARKERS OF SUSCEPTIBILITY

Given the fundamental role of metabolism in toxicological research, increasing attention in the role of genetic variation in toxic responses, and therefore variations in susceptibility and markers of such susceptibility, are of great interest (Timbrell 1998). Initial biomarker research into host factors has been directed at the identification of inter-individual differences in metabolic pathways. A wide range of enzymes that may be associated with disease have been explored, demonstrating substantial differences in levels of activity within the population, such as N-acetyltransferase, several cytochromes P-450 (CYP), and glutathione transferase (GST), among others (Cullen and Redlich 1995, Timbrell 1998, Pavanello and Clonfero 2000). Specifically, trace metals are reported to regulate the expression of CYP as well as heavy metals like Hg and Pb (Ki *et al.* 2009). Each of these enzymes has a potential role in the activation or detoxification of chemical exposures. As the genetic loci of these and other metabolic enzymes have been recognized, the identification of polymorphisms and phenotypic differences in the population has become possible. Polymorphisms and/or acquired differences in enzyme function might be, in part, the cause for differential responses to metals (Cullen and Redlich 1995). As a consequence, the study and identification of single nucleotide polymorphisms (SNPs), becomes essential when studying responses to metal exposures. SNPs are the most abundant forms of DNA sequence variation in the human genome, and contribute to phenotypic diversity, influencing risk of certain diseases, and variable response to the environment (Pavanello and Clonfero 2000).

In the last 20 years, many research groups have been involved on assessing the genotoxic risk of exposed populations according to their genetically determined metabolic characteristics (Timbrell 1998, Pavanello and Clonfero 2000). Unfortunately, in humans most susceptibility studies have focused on infectious diseases or risk factors for cardiovascular disease or cancer and very little attention has been devoted to susceptibility to metal toxicity. Among the few examples analyzing the influence of SNPs on metal exposure responses are: Gundacker *et al.* (2007) analyzing the relationship between polymorphisms in GST genes in individuals exposed to Hg. Also, Tekin *et al.* (2012) determined MT polymorphism in pregnant woman and lead blood levels, concluding that enzyme polymorphisms are well correlated with metal concentrations and with individual susceptibility to toxic effects of metals. For detailed review

TABLE I. DIFFERENCES BETWEEN BIOMARKER RESEARCH APPROACHES FOR ASSESSING ENVIRONMENTAL METAL POLLUTION

Molecules to individuals	Individuals to ecosystems
Usually single compounds	Complex mixtures
High doses, acute exposures	Low doses, chronic exposures
Animal models or occupationally exposed populations	Sentinel species, natural populations
Biomarkers at lower levels of biological organization	Biomarkers at higher levels of biological organization, considering responses at lower levels
Usually non-neutral markers	Usually neutral markers
Concerned with individual and population susceptibility	Concerned with population and ecosystem health
Mechanistic importance	Ecological importance
Time scale decreases	Time scale increases

of MT polimorphisms as biomarkers of individual susceptibility, see Nordberg (1998).

Studies with inorganic arsenic have contributed to a great extent to the knowledge of differences in metal exposure metabolism-responses. Studies concerning the effects of polymorphic forms of arsenic methyl-transferase (AsMT) in regulating the toxicity of AsIII in mice (Stýblo *et al.* 2002, Aposhian *et al.* 2004, Wang *et al.* 2008) highlighted the importance of polymorphisms in the metabolic pathway in mediating formation of toxic methylated arsenical metabolites.

In relation to the susceptibility of lead effect on heme metabolism, several groups have investigated the relationships between ALAD polymorphism and susceptibility to lead toxicity (Schwartz *et al.* 1995, Sakai *et al.* 1996, Alexander *et al.* 1998). These studies concluded that ALAD1 homozygotes might be more susceptible for disturbance in heme biosynthesis than ALAD2 carriers, supported by the fact that ALAD2 protein may bind lead more tightly than ALAD1 protein.

In general, differences in response to heavy metal-associated effects based on genetic variability are not well understood. The only genetic background is far better known for arsenic, mercury and lead, than for the rest of the metals (Gundacker *et al.* 2010).

One reason for the scarce literature on susceptibility of metals is the difficulty of measuring it in isolation; it cannot be separated from other exposures, and controlled exposures are seldom used in humans and difficult to find in natural animal populations.

DEFINITIONS AND TYPES OF BIOMARKERS: FROM INDIVIDUALS TO ECOSYSTEMS

Until now, we have observed that the use of biomarkers at the cellular or sub-cellular levels for analyzing environmental metal exposure is adequate, useful and in some cases, well established. For many years, studies in genetic toxicology and molecular epidemiology have focused on the effects of acute exposures to single toxicants at high doses. Therefore, such biomarkers contribute little to the prediction of the direct consequences for the population in question, hence to the community and ecosystem health. On the contrary, in ecotoxicology threats to populations and communities rising from chronic exposures to mixtures of chemical agents at lower doses (realistic exposures) are the point of interest (Depledge 1994) (**Table I**). Hence, establishing links between cellular and sub-cellular effects and their possible consequences at higher levels of biological organization becomes essential. This is possible by the use of biomarkers in each level of biological organization (**Fig. 1**).

However, incorporation of the biomarker concept in ecotoxicology calls for a redefinition of terms. Definition of biomarkers for ecotoxicology should expand the concept to include changes at the population, community and ecosystem levels, since chemical agents exert their effects at all levels of biological organization. Many studies from the ecotoxicological point of view refer to responses to toxic effects as "*Ecological indicators*" (Cairns and McCormick 1992, Hunsaker 1993). In many other cases, the same responses are regarded as "Biomarkers at population and community levels" where shifts in population and community parameters due to chemical pollution are included (Fossi 1994, Depledge and Fossi 1994, Evenden and Depledge 1997, Moore *et al.* 2004, Bernard 2008). At this point, biomarkers or ecological indicators should give additional information that cannot be obtained from chemical analysis of pollutant concentrations alone, and they may integrate effects of mixtures of chemicals over long exposure periods (Handy *et al.* 2003).

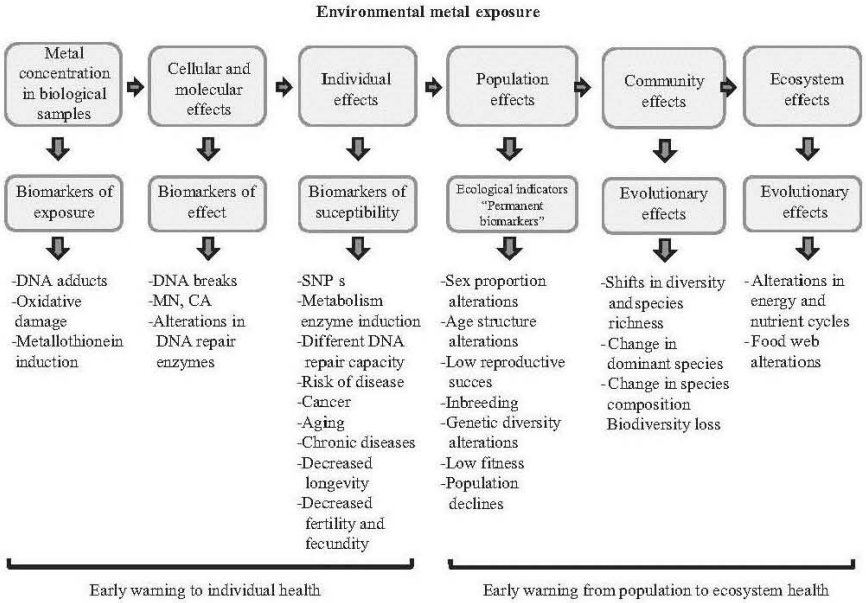

Fig. 1. Environmental pollutants –such as metals– can exert their effects at all levels of biological organization. Most used biomarkers for assessing toxic responses are listed in each level. MN= micronuclei, CA= chromosome aberrations, SNPs= single nucleotide polymorphisms.

Peakall (1994) suggested that when integrating biomarkers to ecotoxicology, three assumptions must be taken into account, since responses to chemical stress from the molecular to the ecosystem level is a continuum of events.

First, the timescale increases, moving from seconds or minutes to years or even decades. Second, the ecological importance increases. Third, it becomes difficult to relate effects to causes as one move up to this continuum (because specific biomarkers for a given chemical agent are more difficult to find). In our opinion, another assumption needs to be taken into account: Mechanistic information about the modes of action of chemical agents is inferred in the lowest levels from this continuum (**Table I, Fig. 2**). Additionally, biomarkers should be chosen so that they reflect changes in the fitness of the population (premature death, ability to mate, fecundity, viability of offspring, etc.) (Evenden and Depledge 1997).

Population level biomarkers

Large phenotypic shifts can evolve in populations over a short period of time. For example, large and rapid evolutionary changes (microevolution) are evident from population responses to pollutants

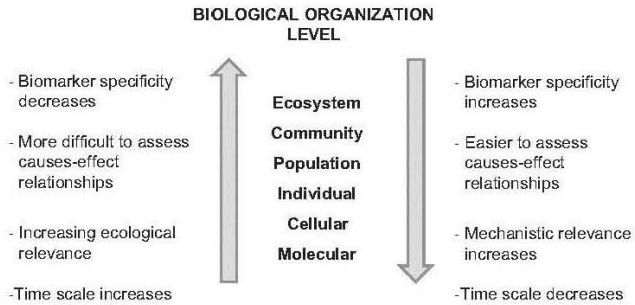

Fig. 2. Assumptions that need to be taken into account when using biomarkers to infer environmental pollution effects at different levels of biological organization. Arrows represent directionality at each level of biological organization for each assumption.

and chemical stress (Luoma 1977). Underlying these microevolutionary changes are shifts in allele frequencies at loci. These changes have long been considered as having potential for monitoring environmental stress.

This process was defined by Medina *et al.* (2007) as "Microevolution due to pollution", it occurs rapidly, in years or after few generations instead of centuries or millennia, involving a variety of physiological, morphological and life-history traits. This fact makes possible to use microevolutionary changes as biomarkers for assessing effects of chemical pollution at the population level (Hoffman and Dabron 2007, Mussali-Galante *et al.* 2012).

Among studies that deal with metal stress populations, the most used approach is to address changes in genetic diversity and allele frequency patterns by using neutral molecular biomarkers (Bickham *et al.* 2000). A neutral biomarker is a sequence of DNA that is polymorphic within a population or a species and that is not under selection. They play an important role in estimating the genetic diversity among individuals by comparing the genotypes at a number of polymorphic loci (Arif and Khan 2009). These markers inform about population demographic processes and have the potential to measure shifts in population size arising from environmental change and adaptation (Bickham and Smolen 1994, Harper-Arabie *et al.* 2004). This fact has led to the hypothesis that neutral markers could be used to monitor pollution effects in populations (Medina *et al.* 2007).

A number of neutral biomarkers which include nuclear and mitochondrial DNA (mtDNA) analyses, such as allozymes, RFLPs (Restriction Fragment Length Polymorphism), SSRs (Simple Sequence Repeats or microsatellite markers), RAPDs (Random Amplified Polymorphic DNA), DNA sequencing of mtDNA, and AFLPs (Amplified Fragment Length Polymorphism) are available with application to genetic ecotoxicological research. However, in the last decade the most used biomarkers to assess genetic diversity in animal populations exposed to metal pollution are: Allozymes, SSR's and mtDNA-sequencing.

Allozyme analysis: This is one of the oldest techniques to assess genetic variability in natural populations. This method analyzes electrophoretic shifts in the charge characteristics of enzymatic proteins produced by amino-acid substitutions. The majority of allozymes show co-dominant inheritance, and the variants are attributed to nucleotide substitutions causing charged amino-acid replacement. This technique detects one-third of amino acid substitution. However, the generally low level of polymorphism at allozyme loci often limits their resolving power in detecting population differences (Diamond *et al.* 1989). Despite their limited resolution, allozyme analysis remains the simplest and most rapid technique for surveying genetic diversity in single copy nuclear genes (Bickham *et al.* 2000). For example, Maes *et al.* (2005) used allozymes and SSRs to analyze allele and genotypic frequencies, levels of polymorphisms and heterozygosity in the European eel (*Anguilla anguilla*) exposed to a mixture of metals (Hg, Cd, Pb, Ni, Cr, As, Se, Cu, Zn). They reported a negative correlation between the level of bioaccumulation and allozymatic multi-locus heterozygosity. Hence, an individual's enzymatic heterozygosity seems to play an important role in the potential to counteract pollutant bioaccumulation. Also, Benton *et al.* (2000) observed decreased heterozygosity in the snail (*Pleurocera canaliculatum*) exposed to Hg using allozymes as biomarkers, reinforcing the use of allozyme analysis as a marker of contamination and possible selection for pollution resistance.

Microsatellites (SSRs): Broadly used for genetic structure and variability analyses, these are short tandem repeats of mono-to tetra-nucleotide repeats which are assumed to be randomly distributed through out the nuclear and mitochondrial genomes. SSRs detects length variation that results from changes in the number of repeat units and their mode of inheritance is co-dominant. Mutations in SSRs are high compared to other DNA markers, therefore, they are considered one of the best molecular markers (Yauk and Quinn 1996, Athrey *et al.* 2007, Tremblay *et al.* 2008) to analyze genetic variability within and between populations. Unfortunately, the identification of SSRs is expensive and requires cloning and sequencing, whilst SSRs primer pairs appear to be species-specific, cross species amplification has been revealed although reduced variability has been observed.

A study conducted by Athrey *et al.* (2007) in which selection for Cd resistance in the least killifish (*Heterandria formosa*) led to increased levels of resistance, but also a decrease in genetic variation as measured by microsatellites. Also, Bourret *et al.* (2008) using SSRs showed that chronic exposure to metal contamination (Cd, Cu) have impacted genetic diversity among populations of the wild yellow perch (*Perca flavescens*), which may affect the capacity of populations to respond to environmental changes. Similar results were obtained by Ungherese *et al.* (2010) who observed decreased genetic diversity in

Hg exposed populations of the sandhopper (*Talitrus saltator*), and by Mussali-Galante *et al.* (2012) in the small mammal (*Peromyscus melanophrys*) exposed to a metal mixture (Pb, As, Cd, Cu, Al).

Mitochondrial DNA analyses: One of the most powerful tools of modern molecular population genetics is nucleotide sequence analysis of mtDNA (Bickham *et al.* 2000). The mitochondrial-protein-coding regions are regarded as powerful markers for genetic diversity analysis. One of the most studied mitochondrial genes in genetic diversity analyses is the cytochrome b, the NADH dehydrogenase and mt-cytochrome oxidase I. Also, the highly polymorphic non-coding region of the mtDNA, termed the control region, has been used in genetic diversity analyses due to its role in replication and transcription of mt-DNA. Advantages of the sequence approach include the ability to target different mitochondrial genes, thus, selecting for targets with an appropriate evolutionary rate as well as the higher resolution obtained by revealing the nucleotide sequence. Moreover, and advantage of the PCR-RFLP analysis of the mt-DNA is that homo and heterozygosity values and allele/genotype frequencies can be determined for the genetic loci analyzed (D'Surney *et al.* 2001, Arif and Kahn 2009). Some illustrative examples include: Matson *et al.* (2006) using mtDNA-sequencing, observed that genetic diversity decreased significantly in exposed populations (Hg) of the marsh frog (*Rana ridibunda*). The authors concluded that environmental degradation due to Hg contamination is the most likely cause of the regional reductions of genetic diversity. On the contrary, Eeva *et al.* (2006) using the same biomarker observed increased nucleotide diversity in populations of the Pied flycatcher (*Parus major)* in polluted sites (Cd, Zn, Cu, Pb, Ni, Al, As, Cr) suggesting high mutation rates. These results are in accordance to various field studies which have demonstrated that mutations accumulate more rapidly in more polluted environments (Yauk and Quinn 1996, Clements 2000, Peles *et al.* 2003, Gardestrom *et al.* 2008).

The majority of studies assessing population genetic responses have observed that populations inhabiting more contaminated environments by heavy metals, hold fairly less genetic diversity, as well as, population differentiation, low reproductive success, reduction of the adaptive potential and lower fitness. Also, these responses have been associated with high levels of DNA damage (Blaise *et al.* 2003, Farag *et al.* 2003). Therefore, a potential association between metal contamination and changes in population genetic structure has been suggested.

Finally, the aforementioned studies clearly illustrate that the concept of biomarkers is successful and deserves a place within the theoretical framework of modern ecotoxicology. Bickham *et al.* (2000) suggested that "because population genetic changes are expected to be independent of the mechanisms of toxicity, and sensitive indicators of transgenerational effects, they represent the ultimate biomarker of effect". Because genetic changes, especially the loss of genetic variability, might be permanent (depending on the population size and mutation rates), once variability is lost the population cannot recover to what it was prior to the environmental impact. Also, there is strong evidence suggesting that genetic population diversity may be a useful biomarker of the health of the ecosystem.

Community level biomarkers

At the community level, changes in composition, richness and species diversity may occur as a consequence of exposure to heavily polluted sites, such as superfund sites, where high levels of heavy metals are found. Due to species interactions, such effects cannot be accurately predicted from effects at the population level, as was recognized by Forbes and Forbes (1993), Hopkin (1993), Smith and Cairns (1993), and Lagadic *et al.* (1994).

Studies assessing community level responses to environmental metal stress are mostly conducted in aquatic ecosystems using invertebrate and fish communities. Among the few studies conducted in terrestrial ecosystems, insect communities are the unit of analysis. For example, Theodorakis *et al.* (2000) analyzed the relationship between biomarkers of effect and changes in fish community structure (diversity and percent pollution-tolerant species) exposed to Hg in sediments. They showed a reduction in species diversity at the most contaminated sites, which tended to increase with increasing distance from the pollution source. They concluded that biomarkers of effect are related to community level responses. Also, Clark and Clements (2006) conducted field and stream microcosm experiments to assess community-level responses (composition, species richness) of macro-invertebrates exposed to heavy metals. They established concentration-response relationships between heavy metals and species richness. Similar results were obtained by Pollard and Yuan (2006) with benthic invertebrate communities along a metal pollution gradient. Moreover, Lefcort *et al.* (2010) found that even after a period of 70 years, heavy metals from mining wastes may still be impacting insect abundance and community structure. Speci-

fically, they found that increased Cd and Zn levels were associated with decreased community diversity.

Ecosystem level biomarkers

At this point, it becomes more difficult to relate ecosystem effects exclusively to metal exposure. Therefore, various authors have recommended more rigorous experimental designs coupled with multidisciplinary research, in order to overcome this problem (Medina *et al.* 2007, Hoffmann and Willi 2008). In spite of this, there are concepts that help to understand ecosystems under chemical stress. Here, some of these concepts are addressed.

Risks to the ecosystem and its components are expected to increase as the amount of pollutant entering the system increases, especially when the ecosystem is polluted by heavy metals, because of their bioaccumulation properties and persistence for long periods of time (Hoffmann and Willi 2008). After the ecosystem health is compromised due to heavy metal pollution, there will be a degree of self-compensation in each ecosystem which will tend to preserve its dynamics somewhat. This is known as ecosystem "resistance" (Moriarty 1999), which is analogous to the compensatory responses exhibited by individual organisms exposed to pollutants (Belfiore and Anderson 1998). A resistant ecosystem may show little change in its dynamics if, for instance, loss of one or more species from the ecosystem following pollutant exposure is associated with replacement by alternative species that serve the same role. However, if key species are lost or mostly impaired, such that ecosystem structure and/or function are affected, then the ensuing ecosystem change shows that ecosystem resistance has been overcome (Moriarty 1999). Interestingly, the replacement of sensitive species by more tolerant species without significant changes in ecosystem structure and function could in itself be interpreted as an "early warning" of a pollutant impact if loss of the species can be directly attributed to exposure to a particular chemical. If biomarkers were to be used to measure toxicity in a sentinel species, population decline might well be detected at an even earlier stage. This illustrates an important principle, namely that monitoring changes in populations of sentinel species might provide a valuable insight into the status of the whole ecosystem (Depledge and Fossi 1994).

Many studies have examined the prevalence and distribution of trace and heavy metals in terrestrial food webs (Hunter and Johnson 1982, Beyer *et al.* 1985, Hunter *et al.* 1987). Patterns of uptake and bioaccumulation have been investigated by studying relationships between metal concentrations in soils and plants and in soils and tissues of co-occurring animals (Sharma and Shupe 1977, Otte *et al.* 1990, Shore 1995). These patterns can reveal general trends of exposure, uptake, translocation, and assimilation of metals within organisms. Trophic transfer of metals within the food web may be demonstrated by relating metal levels in dietary components with those assimilated by an animal (Torres and Jhonson 2001). Finally, bioaccumulation of metals in organisms should be included when analyzing ecosystem effects, since some metal effects may only be recognized in a later phase of life, are multi-generation effects or manifest only in higher members of a food-web. Hence, bioaccumulation of chemicals in biota may be a prerequisite for adverse effects on ecosystems (Van der Oost *et al.* 2003).

IN SEARCH FOR NEW BIOMARKERS OF EXPOSURE TO METAL POLLUTION: THE "OMIC" APPROACH

The field of toxicology has recently begun the process of reinventing itself in view of the rapid technological and conceptual change in molecular biology and genomics. The "omic" approach comprises technologies such as genomics, proteomics, metabolomics, transcriptomics, etc. These new "omics" disciplines apply high-throughput methodologies which changes in expression of hundreds to thousands of genes (genomics), proteins (proteomics) and metabolites (metabolomics) thatare assessed simultaneously (Snape *et al.* 2004). The combination of high-throughput methodologies such as microarray technology and toxicology led to the development of a new scientific discipline, "toxicogenomics" which is the fusion of toxicology, molecular biology, and bioinformatics (Nuwaysir *et al.* 1999). In particular, toxicogenomics offers not just the possibility of determining which molecular pathways are perturbed by toxic compounds, but also a way of exploiting this information, either for the development of new tests or for the development of new biomarkers (Tugwood *et al.* 2003).

The grand goal of toxicology in the post-genome era is to characterize the entire set of genes and proteins that are affected when humans are exposed to environmental xenobiotics. As a consequence, environmental health scientists can conduct large-scale studies of the effects of toxicants on gene expression at the mRNA and protein levels, while simultaneously monitoring metabolite profiles to gain insight into the activity

state of all relevant genes and gene-products. A direct comparison of expression values obtained for a control versus an altered condition reveals a set of biomarkers indicative of that altered state. This exposure "signature" can then be used as a tool for classifying chemical exposures and predicting mode of action (Hamadeh *et al.* 2002, Olden 2006). Specifically for metal exposure assessment, very recently, "metallomics" has emerged as a new sub-discipline of toxicogenomics, which investigates the interrelationships of metal-induced proteome and metabolome changes. In this regard, searches for genes encoding metal-responsive proteins could be interesting targets for reporter genes fusions in biomarker establishing (Haferburg and Kothe 2010).

Furthermore, the integration of the "omic" approach with ecotoxicology, led to the term "ecotoxicogenomics" which includes gene-protein level responses that directly affect population and community dynamics via developmental or reproductive perturbations (Snape *et al.* 2004). Effort towards linking these molecular signatures with alterations in the genetic pool of the affected populations is envisaged. Only then, we will be able to say that "omic" technologies not only help to provide novel biomarkers but also a close look to the continuum of toxic responses from molecules to ecosystems. However, traditional biomarkers targeted for these affected systems should be used to validate the toxic mechanisms of the contaminant. Additionally, one must consider that an expression profile is merely a "snapshot" of a highly dynamic system, and temporal changes in gene and protein expression should be anticipated.

To date, most of the work using DNA microarrays have focused on genetically well characterized organisms, including *Saccharomyces cerevisiae*, *Drosophila melanogaster*, *Caenorhabditis elegans*, *Mus musculus*, and *Homo sapiens*. However, a major obstacle to the application of microarrays in ecotoxicology, is the lack of genomic or cDNA sequence data for sentinel species and non-model organisms (Snape *et al.* 2004, Mehinto *et al.* 2012). In spite of this, gene expression arrays are being developed for a number of non-model organisms; in a variety of fish species (Gracey *et al.* 2001, Jeffries *et al.* 2012), frogs (Altmann *et al.* 2001, Blackshear *et al.* 2001) and birds (Morgan *et al.* 2001, Neiman *et al.* 2001).

One of the best examples when trying to search for new biomarkers of exposure to environmental pollution, was the study conducted by Venier *et al.* (2006) who uncovered over 40 novel biomarkers whose expression levels were regulated similarly in the laboratory and field exposures. Other examples that have illustrated the usefulness of these technologies and have discovered new biomarkers for environmental metal exposures are Wang and Fowler (2008), Ki *et al.* (2009), and Menzel *et al.* (2009).

One of the major disadvantages of gene expression microarrays, is that the analysis of data is complex. There has been some consensus about analysis approaches (Allison *et al.* 2006) but lack of standardization in approaches has introduced difficulties when comparing results between laboratories (Quackenbush 2006).

In spite of these limitations, these novel technologies offer added value compared with classical testing with whole organisms because they provide information concerning the molecular basis of exposure and act as "early warning" signs, enabling a more robust risk assessment than has ever been achieved previously. These new methods might also help to provide data that could reduce much of the uncertainty in extrapolating from laboratory animals to human exposures. Moreover from an ecotoxicological perspective, it is expected that these new methods will provide a better understanding on the application of uncertainty factors that are used to extrapolate data from laboratory to field and from sentinel species to the whole-ecosystem level. More studies are needed to further define the potential applications and limitations of genomics in biomarker research.

CONCLUSIONS AND FINAL REMARKS

From the examples given in these review, it is clear that environmental metal exposure can elicit a plethora of biological effects, ranging from alterations in molecules, compromising individual health and putting ecosystem integrity at risk. Therefore, in each level of biological organization a set of biomarkers can be measured in order to integrate an holistic perspective of complex environmental exposures. Biomarkers at the cellular or sub-cellular levels are adequate, useful and in some cases, well established. However, the use of biomarkers beyond the individual level has not always allowed for cause-effect relationships, since more confounding factors are present, few specific biomarkers are available and often measuring biological responses in field situations becomes difficult.

A major limitation of biomarker use is that a variety of responses have been identified in exposed organisms, making difficult to link environmental exposure to specific chemical entities and subsequent biological effects. In this case, the use of a

multi-biomarker approach, in a range of species using sentinel organisms, becomes necessary to resolve or at least have a closer insight into complex environmental problems. Also, there is a recognized need for biomarker research to move toward a more holistic approach, a proposal that is in harmony with the power of genomics as a tool for understanding toxicant impacts in a diversity of species.

Overall, approaches that integrate responses across levels of organization are especially valuable because they help to understand the mechanistic linkages between the biomarkers responses and the ecologically relevant responses. Therefore, choosing the appropriate biomarker must be based on the biological level of organization in question.

REFERENCES

Abernathy C., Liu Y. and Longfellow D. (1999). Arsenic: health effects, mechanisms of actions, and research issues. Environ. Health Perspect. 107, 593-597.

Ahamed M., Verma S., Kumar A. and Siddiqui M. (2005). Environmental exposure to lead and its correlation with biochemical indices in children. Sci. Total Environ. 346, 48-55.

Ahamed M., Verma S., Kumar A. and Siddiqui M. (2006). Delta-aminolevulinic acid dehydratase inhibition and oxidative stress in relation to blood lead among urban adolescents. Human Exp. Toxicol. 25, 547-553.

Ahnstrom G. (1988). Techniques to measure DNA single-strand breaks in cells: a review. Int. J. Rad. Biol. 54, 695-707.

Aitio A., Bernard A., Fowler A. and Nordberg G. (2007). Biological monitoring and biomarkers. In: *Handbook on the toxicology of metals* (G. Nordberg, B. Fowler, M. Nordberg and L. Friberg, Eds.). 3rd ed. Academic Press/Elsevier, 975 pp.

Al-Sabti K. (1994). Chromium-induced micronuclei in fish. J. Appl. Toxicol. 14, 333-336.

Alexander B., Checkoway H., Costa-Mallen P., Faustman E., Woods J., Kelsey K., van Netten C. and Costa L. (1998). Interaction of blood lead and aminolevulinic acid dehydratase genotype on markers of heme synthesis and sperm production in lead smelter. Environ. Health Perspect. 106, 213-216.

Allison D., Cui X., Page G. and Sabripour M. (2006). Microarray data analysis: from disarray to consolidation and consensus. Nature Rev. Genet. 7, 55-65.

Altenburger R., Scholz S., Schmitt-Jansen M., Busch W. and Escher B. (2012). Mixture toxicity revisited from a toxicogenomic perspective. Environ. Sci. Technol. 46, 2508-2522.

Altmann C., Bell E., Sczyrba A., Pun J., Bekiranov S. and Gaasterland T. (2001). Microarray-based analysis of early development in *Xenopus laevis*. Dev. Biol. 236, 64-75.

Amiard J., Journel R. and Bacheley H. (2008). Influence of field and experimental exposure of mussels (*Mytilus* sp.) to nickel and vanadium on metallothionein concentration. Comp. Biochem. Phys. Part C. 147, 378-385.

Aposhian H., Zakharyan R., Avram M., Sampayo-Reyes A. and Wollenberg L. (2004). A review of the enzymology of arsenic metabolism and a new potential role of hydrogen peroxide in the detoxication of the trivalent arsenic species. Toxicol. Appl. Pharmacol. 198, 327-335.

Arif I. and Khan H. (2009). Molecular markers for biodiversity analysis of wildlife animals: a brief review. Animal Biodiv. Cons. 32, 9-17.

Arrieta M., Bruzzone L., Apartín C., Rosenberg C., Fink N. and Salibián A. (2004). Biosensors of inorganic lead exposure and effect in an adult amphibian. Arch. Environ. Contam. Toxicol. 46, 224-230.

Asmuss M., Mullenders L., Eker A. and Hartwig A. (2000). Differential effects of toxic metal compounds on the activities of Fpg and XPA, two zinc finger proteins involved in DNA repair. Carcinogenesis 21, 2097-2104.

Athrey N., Leberg P. and Klerks P. (2007). Laboratory culturing and selection for increased resistance to cadmium reduce genetic variation in the least killyfish, *Heterandria formosa*. Environ. Toxicol. Chem. 26, 1916-1921.

Augustyniak M., Juchimiuk J., Przybyłowicz W., Mesjasz-Przybyłowicz J., Babczyńska A. and Migula P. (2006). Zinc-induced DNA damage and the distribution of metals in the brain of grasshoppers by the comet assay and micro-PIXE. Comp. Biochem. Physiol. Toxicol. Pharmacol. 144, 242-251.

Baos R., Jovani R., Pastor N., Tella J., Jiménez B. and Gómez G. (2006). Evaluation of genotoxic effects of heavy metals and arsenic in wild nestling white storks (*Ciconia ciconia*) and black kites (*Milvus migrans*) from southwestern Spain after a mining accident. Environ. Toxicol. Chem. 25, 2794-2803.

Basu A., Mahata J., Gupta S. and Giri A. (2001). Genetic toxicology of a paradoxical human carcinogen, arsenic: a review. Mutat. Res. 488, 171-194.

Basu A., Ghosh P., Das J., Banerjee A., Ray K. and Giri A. (2004). Micronuclei as biomarkers of carcinogen exposure in populations exposed to arsenic through drinking water in west Bengal, India: a comparative study in three cell types. Can. Epidemiol. Biomarkers Prev. 13, 8-20.

Bau D., Wang T., Chung C., Wang A. and Jan K. (2002). Oxidative DNA adducts and DNA-protein cross-links are the major DNA lesions induced by arsenite. Environ. Health Perspect. 110, 753-756.

Belfiore S. and Anderson S. (1998). Genetic patterns as a tool for monitoring and assessment of environmental impacts: the example of genetic ecotoxicology. Environ. Monit. Assess. 51, 465-479.

Benton M., Malott M., Trybula J., Dean D. and Guttman S. (2000). Genetic effects of mercury contamination on aquatic snail populations: allozyme genotypes and DNA strand breakage. Environ. Toxicol. Chem. 21, 584-589.

Bernard A. (2008). Biomarkers of metal toxicity in population studies: research potential and interpretation issues. J. Toxicol. Environ. Health A. 71, 1259-1265.

Beyer N., Dalgarn J., Dudding S., French B., Mateo R., Miesner J., Sileo L. and Spann J. (2004). Zinc and lead poisoning in wild bird in the tri-state mining district (Oklahoma, Kansas and Missouri). Arch. Environ. Contam. Toxicol. 48, 108-117.

Beyer W., Pattee O., Sileo L., Hoffman D. and Mulhern B. (1985). Metal contamination in wildlife living near two zinc smelters. Environ. Pollut. Ser. 38, 63-86.

Beyersmann D. (2002). Effects of carcinogenic metals on gene expression. Toxicol. Lett. 127, 63-68.

Bickham J. and Smolen M. (1994). Somatic and heritable effects of environmental genotoxins and the emergence of evolutionary toxicology. Environ. Health Perspect. 102, 25-28.

Bickham J., Sandhu S., Hebert P., Chikhi L. and Athwal R. (2000). Effects of chemical contaminants on genetic diversity in natural populations: implications for biomonitoring and ecotoxicology. Mutat. Res. 463, 33-51.

Bilban M. (1998). Influence of the work environment in a Pb-Zn mine on the incidence of cytogenetic damage in miners. Am. J. Ind. Med. 34, 455-463.

Bjorkman L., Vahter M. and Pedersen N. (2000). Both the environment and genes are important for concentrations of cadmium and lead in blood. Environ. Health Perspect. 36, 1081-1086.

Blackshear P., Lai W., Thorn J., Kennington E., Staffa N. and Moore D. (2001). The NIEHS *Xenopus* maternal EST project: interim analysis of the first 13,879 ESTs from unfertilized eggs. Gene 267, 71-87.

Blaise C., Gagne F. and Pellerin J. (2003). Bivalve population status and biomarker responses in *Mya arenaria* clams (Saguenay Fjord, Quebec, Canada). Fresenius Environ. Bull. 12, 956-960.

Boei J. and Natarajan A. (1995). Detection of chromosome malsegregation to the daughter nuclei in cytokinesis-blocked transgenic mouse splenocytes. Chromosome Res. 3, 45-53.

Bolognesi C. and Hayashi M. (2011). Micronucleus assay in aquatic animals. Mutagenesis 26, 205-213.

Bonassi S., Ugolini D., Kirsch-Volders M., Stromberg U., Vermeulen R. and Tucker J. (2005). Human population studies with cytogenetic biomarkers: review of the literature and future prospective. Environ. Mol. Mutagen. 45, 258-270.

Bourret V., Couture P., Campbell P. and Bernatchez L. (2008). Evolutionary ecotoxicology of wild yellow perch (*Percha flavescens*) populations chronically exposed to a polymetallic gradient. Aquat. Toxicol. 86, 76-90.

Cadet J., Douki T., Frelon S., Sauvaigo S., Pouget J. and Ravanat J. (2002). Assessment of oxidative base damage to isolated and cellular DNA by HPLC-MS/MS measurement. Free Radical Biol. Med. 33, 441-449.

Cairns J. and McCormick E. (1992). Developing an ecosystem-based capability for ecological risk assessments. Environ. Profession. 14, 186-196.

Calderón J., Ortiz-Pérez D., Yañez L. and Díaz-Barriga F. (2003). Human exposure to metals. Pathways of exposure, biomarkers of effect, and host factors. Ecotoxicol. Environ. Saf. 56, 93-103.

Chen H., Rupa D., Tomar R. and Eastmond D. (1994). Chromosomal loss and breakage in mouse bone marrow and spleen cells exposed to benzene in vivo. Cancer Res. 54, 3533-3539.

Chung J., Nii O., Nartey D. and Cheria M. (1986). Metallothionein levels in liver and kidney of Canadians potential indicator of environmental exposure to cadmium. Arch. Environ. Health. 41, 319-323.

Clark J. and Clements W. (2006). The use of in situ and stream microcosm experiments to assess population and community-level responses to metals. Environ. Toxicol. Chem. 25, 2306-2312.

Clarkson T., Friberg L., Nordberg G. and Sager R. (1988). *Biological monitoring of toxic metals.* Plenum Press, New York, 686 pp.

Clements W. (2000). Integrating effects of contaminants across levels of biological organization: an overview. J. Aquat. Ecosyst. Stress Recovery. 7, 113-116.

Costa P., Repolho T., Caeiro S., Diniz E., Moura I. and Costa M. (2008). Modelling metallothionein induction in the liver of *Sparus aurata* exposed to metal-contaminated estuarine sediments. Ecotoxicol. Environ. Saf. 71, 117-124.

Costa P., Caeiro S., Mario S., Diniz E., Martins M., Ferreira A., Caetano M., Vale C., Del Valls H. and Costa M. (2009). Biochemical endpoints on juvenile *Solea senegalensis* exposed to estuarine sediments: the effect of contaminant mixtures on metallothionein and CYP1A induction. Ecotoxicol. 18, 988-1000.

Cullen M. and Redlich C. (1995). Significance of individual sensitivity to chemicals: elucidation of host susceptibility by use of biomarkers. Clin. Chem. 41, 1809-1813.

De Flora S. and Wetterhahn K. (1989). Mechanisms of chromium metabolism and genotoxicity. Life Chem. Rep. 7, 169-244.

Depledge M. (1994). Genotypic toxicity: implications for individuals and populations. Environ. Health Perspect. 102, 101-104.

Depledge M. and Fossi M. (1994). The role of biomarkers in environmental assessment. Invert. Ecotoxicol. 3, 161-172.

Dhawan A., Bajpayee M. and Parmar D. (2009). Comet assay: A reliable tool for the assessment of DNA damage in different models. Cell Biol. Toxicol. 25, 5-32.

Diamond S., Newman M., Mulvey M., Dixon P. and Martinson D. (1989). Allozyme genotype and time to death of mosquito fish, *Gambusia affinis* (Baird and Girard), during acute exposure to inorganic mercury. Environ. Toxicol. Chem. 8, 613-622.

Dizdaroglu M. (1992). Oxidative damage to DNA in mammalian chromatin. Mutat. Res. 275, 331-342.

D'Surney S., Shugart L. and Theodorakis C. (2001). Genetic markers and genotyping methodologies: an overview. Ecotoxicol. 10, 201-204.

Eeva T., Belskii E. and Kuranov B. (2006). Environmental pollution affects genetic diversity in wild bird populations. Mutat. Res. 608, 8-15.

Ehrenberg L., Granath F. and Törnqvist M. (1996). Macromolecule adducts as biomarkers of exposure to environmental mutagens in human populations. Environ. Health Perspect. 104, 423-428.

Ehrlich V., Nersesyan A., Hoelz C., Ferk F., Bichler J., Valic E., Schaffer A., Schulte-Hermann R., Fenech M., Wagner K. and Knasmüller S. (2008). Inhalative exposure to vanadium pentoxide causes DNA damage in workers: results of a multiple end point study. Environ. Health Perspect. 116, 1689-1693.

EPA (2000). *Innovative remediation technologies: field scale demonstration projects in North America*. 2nd Ed. Environmental Protection Agency. National Service Center for Environmental Publications (NSCEP), 139 pp.

Evenden A. and Depledge M. (1997). Genetic susceptibility in ecosystems: the challenge for ecotoxicology. Environ. Health Perspect. 105, 849-854.

Fan S., Ho I., Yeoh F., Lin C. and Lee T. (1996). Squalene inhibites sodium arsenite induced sister chromatid exchanges and micronuclei in Chinese hamster ovary K1 cells. Mutat. Res. 368, 165-169.

Farag A., Skaar D., Nimick D., MacConnell E. and Hogstrand C. (2003). Characterizing aquatic health using salmonid mortality, physiology, and biomass estimates in streams with elevated concentrations of arsenic, cadmium, copper, lead, and zinc in the Boulder River watershed, Montana. Trans. Am. Fish Soc. 132, 450-467.

Farmer P. (2004). DNA and protein adducts as markers of genotoxicity. Toxicol. Lett. 149, 3-9.

Fenech M., Holland N., Chang W., Zeiger E. and Bonassi S. (1999). The human micro-nucleus project-an international collaborative study on the use of the micronucleus technique for measuring DNA damage in humans. Mutat. Res. 428, 271-283.

Fenech M. and Crott J. (2002). Micronuclei, nucleoplasmic bridges and nuclear buds induced in folic acid deficient human lymphocytes evidence for breakage-fusion-bridge cycles in the cytokinesis-block micronucleus assay. Mutat. Res. 504, 131-136.

Filipic M. and Hei T. (2004). Mutagenicity of cadmium in mammalian cells: implication of oxidative DNA damage. Mutat. Res. 546, 81-91.

Fogu G., Congiu A., Campus P., Ladu R., Sanna R., Sini M. and Soro G. (2000). Genotoxic effects of cadmium chloride on human amniotic fluid cells cultured in vitro. Ann. Chim. 90, 709-714.

Forbes T. and Forbes V. (1993). A critique of the use of distribution-based extrapolation models in ecotoxicology. Funct. Ecol. 7, 249-254.

Fossi M. (1994). Nondestructive biomarkers in ecotoxicology. Environ. Health Perspect. 102, 49-54.

Fourie F., Reinecke S. and Reinecke A. (2007). The determination of earthworm species sensitivity differences to cadmium genotoxicity using the comet assay. Ecotoxicol. Environ. Saf. 67, 361-368.

Fowler B. (1987). Intracellular compartmentation of metals in aquatic organisms: roles and mechanisms of cell injury. Environ. Health Perspect. 71, 121-128.

Fowler B. (1992). Mechanisms of kidney cell injury from metals. Environ. Health Perspect. 100, 57-63.

Fowler B., Chou C., Jones R. and Chen C. (2007). Arsenic. In: *Handbook on the toxicology of metals* (G. Nordberg, B.A. Fowler, M. Nordberg and L. Friberg, Eds.). Elsevier, Amsterdam, pp. 367- 406.

Frenzilli G., Nigroa M. and Lyons B. (2009). The comet assay for the evaluation of genotoxic impact in aquatic environments. Celebrating the 20th Anniversary of the invention of the comet assay. Mutat. Res. Rev. 681, 80-92.

Gallo V., Khan A., Gonzales C., David H., Schoket B. and Vineis P. (2008). Validation of biomarkers for the study of environmental carcinogens: a review. Biomarkers 13, 505-534.

Gardestrom J., Dahl U., Kotsalainen K., Maxson A., Elfwing T., Grahn M., Bengtsson B. and Breitholtz M. (2008).

Evidence of population genetic effects of long-term exposure to contaminated sediments: a multi-endpoint study with copepods. Aquat. Toxicol. 86, 426-436.

Gauthier J., Dubeau H., Rassart E., Jarman W. and Wells R. (1999). Biomarkers of DNA damage in marine mammals. Mutat. Res. 444, 427-439.

Gennart P., Baleu C., Verellen-Dumoulin C., Buchet P., De Meyer R. and Lauwerys R. (1993). Increased sister chromatid exchanges and tumor markers in workers exposed to elemental chromium-cobalt- and nickel-containing dusts. Mutat. Res. 299, 55-61.

George G. and Olsson E. (1994). Metallothioneins as indicators of trace metal pollution. In: *Biomonitoring in coastal waters and estuaries* (K. Kramer Ed.). CRC Press, Boca Raton, pp. 151-178.

Gonsebatt M., Vega L., Salazar A., Montero R., Guzmán P., Blas J., Del Razo L., García-Vargas G., Albores A. and Cebrián M. (1997). Cytogenetic effects in human exposure to arsenic. Mutat. Res. 386, 219-228.

Gracey A., Troll J. and Somero G. (2001). Hypoxia-induced gene expression profiling in the euryoxic fish *Gillichthys mirabilis*. Proc. Natl. Acad. Sci. USA 98, 1993-1998.

Gummow B., van den Broek J., Kirsten W., Botha C., Noordhuizen J. and Heesterbeek J. (2006). The assessment of biomarkers in sentinel cattle for monitoring vanadium exposure. J. Environ. Monit. 8, 445-455.

Gundacker C., Komarnicki G., Jagiello P., Gencikova A., Dahmen N., Wittmann C. and Gencik M. (2007). Glutathione-S-transferase polymorphism, metallothionein expression, and mercury levels among students in Austria. Sci. Total Environ. 385, 37-47.

Gundacker C., Gencik M. and Hengstschlager M. (2010). The relevance of the individual genetic background for the toxicokinetics of two significant neurodevelopmental toxicants: mercury and lead. Mutat. Res. 705, 130-140.

Gurgueira S., Lawrence J., Coull B., Murthy G. and Gonzalez-Flecha G. (2002). Rapid increases in the steady-state concentration of reactive oxygen species in the lungs and heart after particulate air pollution inhalation. Environ. Health Perspect. 110, 749-755.

Haferburg G. and Kothe E. (2010). Metallomics: lessons for metalliferous soil remediation. Appl. Microbiol. Biotech. 87, 1271-1280.

Hagmar L., Brogger A., Hansteen I.L., Heim S., Hogstedt B., Knudsen L., Lambert B., Linnainmaa K., Mitelman F. and Nordenson I. (1994). Cancer risk in humans predicted by increased levels of chromosomal aberrations in lymphocytes: nordic study group on the health risk of chromosome damage. Cancer Res. 54, 2919-2922.

Halliwell B. and Gutteridge J. (1999). *Free radicals in biology and medicine*. 3rd ed. Oxford University Press, Oxford, 936 pp.

Hamadeh H., Bushel P., Jayadev S., Martin K., DiSorbo O., Sieber S., Bennett L., Tennant R., Stoll R., Barrett J., Blanchard K., Paules R. and Afshari C. (2002). Gene expression analysis reveals chemical-specific profiles. Toxicol. Sci. 67, 219-231.

Handy R., Galloway T. and Depledege M. (2003). A proposal for the use of biomarkers for the assessment of chronic pollution and in regulatory toxicology. Ecotoxicology 12, 331-343.

Harper-Arabie R., Wirth E., Fulton M. and Ross P. (2004). Protective effects of allozyme genotype during chemical exposure in the grass shrimp, *Palaemonetes pugio*. Aquat. Toxicol. 70, 41-54.

Hartmann A. and Speit G. (1994). Comparative investigations of the genotoxic effects of metals in the single cell gel (SCG) assay and the sister chromatid exchange (SCE) test. Environ. Mol. Mutagenesis. 23, 299-305.

Hartwig A., Goblinghoff U., Beyersmann D., Natarajan A., Filon R. and Mullenders L. (1997). Interaction of arsenic(III) with neuclotide excision repair in UV irradiated human fibroblasts. Carcinogenesis 18, 399-405.

Hartwig A. (1998). Carcinogenicity of metal compounds: possible role of DNA-repair inhibition. Toxicol. Lett. 102-103, 235-239.

Hartwig A. (2001). Zinc finger proteins as potential targets for toxic metal ions: differential effects on structure and function. Antiox. Redox Sign. 3, 625-634.

Hartwig A., Asmuss M., Ehleben I., Herzer U., Kostelac D. and Pelzer A. (2002). Interference by toxic metal ions with DNA repair processes. Env. Health Perspect. 110, 797-799.

Hayes R. (1997). The carcinogenicity of metals in humans. Can. Causes Control 8, 371-385.

Hebert P. and Murdoch M. (1996). Genetic effects of contaminant exposure-towards an assessment of impacts on animal populations. Sci. Total Environ. 191, 23-58.

Hoffman A. and Dabron P. (2007). Towards genetic markers in animal populations as biomonitors for human induced environmental change. Ecol. Lett. 10, 63-76.

Hoffmann A. and Willi Y. (2008). Detecting genetic responses to environmental change. Nature Rev. Geneto. 9, 421-431.

Hooftman R. and Vink G. (1981). Cytogenetic effects on the eastern mudminnow, *Umbra pygmaea*, exposed to ethyl methane sulfonate, benzo[a]pyrene, and river water. Ecotoxicol. Environ. Saf. 5, 261-269.

Hopkin S. (1993). Ecological implications of 95% protection levels for metals in soil. Oikos 66, 137-141.

Hughes M. (2006). Biomarkers of exposure: a case study with inorganic arsenic. Environ. Health Pers. 114, 360-369.

Hunsaker C. (1993). New concepts in environmental monitoring: the question of indicators. Sci. Total Environ. 101, 77-95.

Hunter B. and Johnson S. (1982). Food chain relationships of copper and cadmium in contaminated grassland ecosystems. Oikos 38, 108-117.

Hunter B., Johnson S. and Thompson D. (1987). Ecotoxicology of copper and cadmium in a contaminated grassland ecosystem. Soil and vegetation contamination. J. Appl. Ecol. 24, 573-586.

Jakubowski M. and Trzcinka-Ochocka M. (2005). Biological monitoring of exposure: trends and key developments. J. Occup. Health. 47, 22-48.

Jeffries M., Mehinto A., Carter B., Denslow N. and Kolok A. (2012). Taking microarrays to the field: differential hepatic gene expression of caged fathead minnows from Nebraska watersheds. Environ. Sci. Technol. 46, 1877-1885.

Johnson D., Audet J., Kern W., LeCaptain J., Strickland D., Hoffman J. and McDonald L. (1999). Lead exposure in passerines inhabiting lead-contaminated floodplains in the Coeur d'Alene River Basin, Idaho, USA. Environ. Toxicol. Chem. 18, 1190-1194.

Kaina B. (2004). Mechanisms and consequences of methylating agent induced SCEs and chromosomal aberrations: a long road traveled and still a far way to go. Cytogenet. Genome Res.104, 77-86.

Kato H. (1974). Spontaneous sister chromatid exchanges detected by a BUdR-labelling method. Nature. 251, 70-72.

Kawanishi S., Hiraku Y. and Oikawa S. (2001). Mechanism of guanine-specific DNA damage by oxidative stress and its role in carcinogenesis and aging. Mutat. Res. 488, 65-76.

Ki J., Raisuddin S., Lee K., Hwang D., Han D., Rhee J., Kim I., Park H., Ryu J. and Lee J. (2009). Gene expression profiling of copper-induced responses in the intertidal copepod *Tigriopus japonicus* using a 6K oligochip microarray. Aquat. Toxicol. 93, 177-187.

Koch C., Giandomenico A. (1994). The alkaline elution technique for measuring DNA single strand breaks: Increased reliability and sensitivity. Anal. Biochem. 220, 58-65.

Kuo H., Chang F., Wu Y. and Wu Y. (2003). Chromium (VI) induced oxidative damage to DNA: increase of urinary 8-hydroxydeoxyguanosine concentrations (8-OHdG) among electroplating workers. Occup. Environ. Med. 60, 590-594.

Lagadic L., Caquet T. and Ramade F. (1994). The role of biomarkers in environmental assessment. invertebrate populations and communities. Ecotoxicology. 3, 193-208.

Lai J., Kuo W., Liao F. and Lien C. (1998). Sister chromatid exchange induced by chromium compounds in human lymphocytes. Int. Arch. Occup. Environ. Health. 71, 550-553.

Langston J., Bebianno J. and Burt R. (1998). Metal handling strategies in molluscs. In: *Metal metabolism in aquatic environments* (J. Langston and Bebianno J. Eds.). Chapman and Hall, London, pp. 219-283.

Latt S. (1973). Microfluorometric detection of deoxyribonucleic acid replication in human metaphase chromosomes. Proc. Natl. Acad. Sci. 70, 3395-3399.

Lee H., Lim S., Song K., Boo Y. and Jacobs R. (2006). Graded association of blood lead and urinary cadmium concentrations with oxidative stress related markers in the U.S. population: results from the third national health and nutrition examination survey. Environ. Health Perspect. 114, 350-354.

Lefcort H., Vancura J. and Lider E. (2010). 75 years after mining ends stream insect diversity is still affected by heavy metals. Ecotoxicology 19, 1416-1425.

Leon G., Espitia-Pérez L. and Linares J. (2007). Genotoxic effects in wild rodents (*Rattus rattus* and *Mus musculus*) in an open coal mining area. Mutat. Res. 630, 42-49.

Lerda D. (1994). Sister chromatid exchange (SCE) among individuals chronically exposed to arsenic in drinking water. Mutat. Res. 312, 111–120.

Limón-Pacheco L. and Gonsebatt M. (2009). The role of antioxidants and antioxidant-related enzymes in protective responses to environmentally induced oxidative stress. Mutat. Res. 674, 37-147.

Lindberg H., Wang X., Jarventaus H., Ghita C., Falck M., Norppa M. and Fenech M. (2007). Origin of nuclear buds and micronuclei in normal and folate-deprived human lymphocytes. Mutat. Res. 617, 33-45.

Link S., Kensler J. and Groopman, J. (1995). Biomarkers and mechanistic approaches in environmental epidemiology. Ann. Rev. Publ. Health. 16, 83-103.

Loft S. and Poulsen H. (1999). Markers of oxidative damage to DNA: antioxidants and molecular damage. Methods Enzymol. 300, 166-184.

Lu J., Jin T., Nordberg G. and Nordberg M. (2005). Metallothionein gene expression in peripheral lymphocytes and renal dysfunction in a population environmentally exposed to cadmium. Toxicol. Appl. Pharmacol. 206, 150-156.

Luoma S. (1977). Detection of trace contaminant effects in aquatic ecosystems. J. Fish Res. Bd. Can. 34, 436-439.

Machella N., Battino M., Pisanelli B. and Regoli F. (2006). Influence of the SCGE protocol on the amount of basal DNA damage detected in the Mediterranean mussel, *Mytilus galloprovincialis*. Environ. Mol. Mutagen. 47, 579-586.

Maes G., Raeymaekers J., Pampoulie C., Seynaeve A., Goemans G., Belpaire C. and Volckaert F. (2005). The catadromous European eel *Anguilla anguilla* (L.) as a model for freshwater evolutionary ecotoxicology: relationship between heavy metal bioaccumulation, condition and genetic variability. Aquat. Toxicol. 73, 99-114.

Mao Y., Liu K., Jiang J. and Shi X. (1996). Generation of reactive oxygen species by Co (II) from H_2O_2 in the presence of chelators in relation to DNA damage and 2-deoxyguanosine hydroxylation. J. Toxicol. Environ. Health 47, 61-75.

Martinez E., Sousa A., María-Mojica P., Gómez-Ramírez P., Guilhermino L. and García-Fernández G. (2010). Blood d-ALAD, lead and cadmium concentrations in spur-thighed tortoises (*Testudo graeca*) from south-eastern Spain and northern Africa. Ecotoxicology 19, 670-677.

Matson C., Megan M., Lambert L., McDonald T., Autenrieth R., Donnelly K., Islamzadeh A., Politov D. and Bickham J. (2006). Evolutionary toxicology: population-level effects of chronic contaminant exposure on the marsh frogs (*Rana ridibunda*) of Azerbaijan. Environ. Health Perspect. 114, 547-552.

McBee K., Bickham J., Brown K. and Donnelly K. (1987). Chromosomal aberrations in native small mammals (*Peromyscus leocopus* and *Sigmodon hispidus*) at a petrochemical waste disposal site. I. Standard karyology. Arch. Environ Contam. Toxicol. 16, 681-688.

McBee K. and Bickham J. (1988). Petrochemical-related DNA damage in wild rodents detected by flow cytometry. Bull. Environ. Contam. Toxicol. 40, 433-449.

Mehinto A., Martyniuk C., Spade D. and Denslow N. (2012). Applications for next-generation sequencing in fish ecotoxicogenomics. Front. Genet. 3, 62-70.

Medina J., Correa C. and Barata J. (2007). Micro-evolution due to pollution: possible consequences for ecosystem responses to toxic stress. Chemosphere 67, 2105-2114.

Méndez-Gómez J., García-Vargas G., López-Carrillo L., Calderón-Aranda E., Gómez A., Vera E., Valverde M., Cebrián M. and Rojas E. (2008). Genotoxic effects of environmental exposure to arsenic and lead on children in region lagunera, Mexico. Ann. Acad. Sci. 1140, 358-367.

Menzel R., Swain S., Hoess S., Claus E., Menzel S., Steinberg C., Reifferscheid G. and Stürzenbaum R. (2009). Gene expression profiling to characterize sediment toxicity: a pilot study using *Caenorhabditis elegans* whole genome microarrays. BMC Genomics 10, 160-170.

Michailova P., Petrova N., Ramella L., Sella G., Todorova J. and Zelano V. (1996). Cytogenetic characteristics of a population of *Chironomus riparius* Meigen 1804 (Diptera, Chironomidae) from a polluted Pb river station. Genetica 98, 55-62.

Minissi S., Ciccotti E. and Rizzoni M. (1996). Micronucleus test in erythrocytes of *Barbus plebejus* (Teleostei, Pisces) from two natural environments: a bioassay for the in situ detection of mutagens in freshwater. Mutat. Res. 367, 245-251.

Moore M., Depledge M., Readmana J. and Leonard D. (2004). An integrated biomarker-based strategy for ecotoxicological evaluation of risk in environmental management. Mutat. Res. Rev. 552, 247-268.

Morgan R., Sofer L., Anderson A., Bernberg E., Cui J. and Burnside J. (2001). Induction of host gene expression following infection of chicken embryo fibroblasts with oncogenic Marek's disease virus. J. Virol. 75, 533-539.

Moriarty F. (1999). *Ecotoxicology, the study of pollutants in ecosystems*. Academic Press. London, UK, 347 pp.

Mouron S., Grillo C., Dulout F. and Golijow C. (2004). A comparative investigation of DNA strand breaks, sister chromatid exchanges and K-ras gene mutations induced by cadmium salts in cultured human cells. Mutat. Res. 568, 221-231.

Mukherjee A., Kumar Giri A., Sharma A. and Talukder G. (1988). Relative efficacy of short-term tests in detecting genotoxic effects of cadmium chloride in mice in vivo. Mutat. Res. 206, 285-295.

Muller J., Kayser L., Paikoff S., Duarte V., Tang N., Perez R., Rokita S. and Burrows C. (1999). Formation of DNA adducts using nickel(II) complexes of redox-active ligands: a comparison of salen and peptide complexes. Coord. Chem. Rev. 186, 761-774.

Mussali-Galante P., Ávila-Costa M., Piñón-Zarate G., Martínez-Levy G., Rodríguez-Lara V. and Rojas-Lemus M. (2005). DNA damage as an early biomarker of effect in human health. Toxicol. Ind. Health. 21, 55-66.

Mussali-Galante P., Tovar-Sánchez E. and Fortoul T. (2007). Cell cycle, P53 and metals. In: *Metals and Toxicological Implications in Health* (I.T. Fortoul, Ed.). Research Signpost. Kerala, India, pp. 9-13.

Mussali-Galante P., Tovar-Sánchez E., Valverde M., Valencia-Cuevas L. and Rojas E. (2012). Evidence of population genetic effects in *Peromyscus melanophrys* chronically exposed to mine tailings in Morelos, Mexico. Environ. Sci. Pollut. Res. doi 10.1007/s11356-012-1263-8.

Mutti A. (1995). Use of intermediate end-points to prevent long-term outcomes. Toxicol. Lett. 77, 121-125.

Natarajan A. (1976). Molecular aspects of the origin of chromosome structural changes. Biol. Zbl. 95, 139-156.

Neiman P., Ruddell A., Jasoni C., Loring G., Thomas S. and Brandvold K. (2001). Analysis of gene expression

during myc oncogene-induced lymphomagenesis in the bursa of Fabricius. Proc. Natl. Acad. Sci. USA 98, 6378-6383.

Nordberg G. (1998). Metallothioneins: historical review and state of knowledge. Talanta 46, 243-254.

Nordberg G., Nogawa K., Nordberg M. and Friberg L. (2007). Cadmium. In: *Handbook on the Toxicology of Metals* (G. Nordberg, Fowler B., Nordberg M. and Friberg L. Eds.). 3rd ed. Academic Press/Elsevier, pp. 446-486.

Nordberg G. (2010). Biomarkers of exposure, effects and susceptibility in humans and their application in studies of interactions among metals in China. Toxicol. Letts. 192, 45-49.

NRC (1987). Committee on biological markers of the National Research Council. Biological markers in environmental health research. Eviron. Health. Perspect. 74, 3-9.

NRC (1989). *Biological markers in reproductive toxicology*. National Research Council. Washington, DC. National Academy Press, 420 pp.

Nuwaysir F., Bittner M., Trent J., Barrett C. and Afshari C. (1999). Microarrays and toxicology: the advent of toxicogenomics. Mol. Carcinog. 24, 153-159.

Obe G., Pfeiffer P., Savage J., Johannes C., Goedecke W., Jeppesen P., Natarajan A., Martinez-López A., Folle G. and Drets M. (2002). Chromosomal aberrations: formation, identification and distribution. Mutat. Res. 504, 17-36.

Ohno H., Hanaoka F. and Yamada M. (1982). Inducibility of sister chromatid exchanges by heavy metal ions. Mutat. Res. 104, 141-145.

Olden K. (2006). Toxicogenomics-A new systems toxicology approach to understanding of gene-environment interactions. Ann. NY Acad. Sci. 1076, 703-706.

Olsvik P., Hindar K., Zachariassen K., Rolf A. and Andersen A. (2001). Brown trout (*Salmo trutta*) metallothioneins as biomarkers for metal exposure in two Norwegian rivers. Biomarkers 6, 274-288.

Ostrosky-Wegman P., Gonsebat M., Montero R., Vega L., Barba H., Espinosa J., Palao A., Cortinas C., García-Vargas G. and Del Razo L. (1991). Lymphocyte proliferation kinetics and genotoxic findings in a pilot study on individuals chronically exposed to arsenic in Mexico. Mutat. Res. 250, 477-482.

Otte M., Rozema J., Beek M., Kater B. and Broekman R. (1990). Uptake of arsenic by estuarine plants and interactions with phosphate, in the field (Rhine Estuary), and under outdoor experimental conditions. Sci. Total Environ. 97-98, 839-854.

Pandey P., Yadav S. and Pandey M. (2007). Human arsenic poisoning issues in central-east Indian locations: biomarkers and biochemical monitoring. Int. J. Environ. Res. Public Health 4, 15-22.

Pastor N., López-Lázaro M., Tella J., Baos R., Forrero M., Hiraldo F. and Cortés F. (2001). DNA damage in birds alters the mining waste spill in southwestern Spain: a comet assay evaluation. J. Environ. Pathol. Toxicol. Oncol. 20, 317-324.

Pastor N., López-Lázaro M., Tella J., Baos R., Forrero M., Pastor N., Baos R., López-Lázaro M., Jovani R., Tella J. and Hajji N. (2004). A 4 year follow-up analysis of genotoxic damage in birds of the Doñana area (south west Spain) in the wake of the 1998 mining waste spill. Mutagenesis 19, 61-65.

Pavanello S. and Clonfero E. (2000). Biological indicators of genotoxic risk and metabolic polymorphisms. Mutat. Res. 463, 285-308.

Peakall D. and Shugart L. (1992). *Biomarkers: Research and application in the assessment of Environ Health*. Berlin and Heidelberg, Springer 199 p.

Peakall D. (1994). The role of biomarkers in environmental assessment. Ecotoxicology 3, 157-160.

Peles D., Towler W. and Guttman S. (2003). Population genetic structure of earthworms (*Lumbricus rubelluz*) in soils contaminated by heavy metals. Ecotoxicology. 12, 379-386.

Perceval O., Couillard Y., Pinel-Alloul P., Giguerec A. and Campbell P. (2004). Metal-induced stress in bivalves living along a gradient of Cd contamination: relating sub-cellular metal distribution to population-level responses. Aquat. Toxicol. 69, 327-345.

Perera F. (1996). Molecular epidemiology: insights into cancer susceptibility. Risk assess prevention. J. Natl. Can. Inst. 88, 496-509.

Perera F. (2000). Molecular epidemiology: on the path to prevention? J. Natl. Can. Inst. 92, 602-612.

Perera F. and Weinstein B. (2000). Molecular epidemiology, recent advances and future perspectives. Carcinogenesis 21, 517-524.

Perry P. and Wolf S. (1974). New Giemsa method for the differential staining of sister chromatid. Nature 251, 156-158.

Petering D. and Fowler B. (1986). Roles of metallothionein and related proteins in metal metabolism and toxicity: problems and perspectives. Environ. Health Perspect. 65, 217-224.

Pfeiffer P., Goedecke W. and Obe G. (2000). Mechanisms of DNA double-strand break repair and their potential to induce chromosomal aberrations. Mutagenesis 15, 289-302.

Poirier M. (2004). Chemical-induced DNA damage and human cancer risk. Nat. Rev. Can. 4, 630-637.

Pollard A. and Yuan L. (2006). Community response patterns: evaluating benthic invertebrate composition in metal-polluted streams. Ecol. Appl. 16, 645-655.

Prein A., Thie G., Alink G., Koeman J. and Poels C. (1978). Cytogenetic changes in fish exposed to water of the river Rhine. Sci. Total Environ. 9, 287-291.

Quackenbush J. (2006). Computational approaches to analysis of DNA microarray data. Methods Inf. Med. 45, 91-103.

Randerath K., Moorthy B., Mabon N. and Sriam P. (1994). Tamoxifen: evidence by ^{32}P-postlabeling and use of metabolic inhibitor for two distinct pathway leading to mouse hepatic DNA adduct formation and identification of 4-hydroxytamoxifen as a proximate metabolite. Carcinogenesis 15, 2087-2094.

Reinecke S. and Reinecke A. (2004). The comet assay as biomarker of heavy metal genotoxicity in earthworms. Arch. Environ. Contam. Toxicol. 46, 208-215.

Rodríguez-Mercado J., Roldán-Reyes E. and Altamirano M. (2003). Genotoxic effects of vanadium (IV) in human peripheral blood cells. Toxicol. Lett. 144, 359-369.

Rodríguez-Mercado J. and Altamirano-Lozano M. (2006). Vanadio: contaminación, metabolismo y genotoxicidad. Rev. Int. Contam. Ambient. 22, 173-189.

Rojas E., López M. and Valverde M. (1999). Single cell gel electrophoresis assay: methodology and applications. J. Chromatogr. B. 722, 225-254.

Rojas E. (2009). Special issue on the 20th anniversary of the comet assay. Mutat. Res. Rev. 681, 1-2.

Romero-Isart N. and Vasak M. (2002). Advances in the structure and chemistry of metallothioneins. J. Inorg. Biochem. 88, 388-396.

Rossman T. (2003). Mechanism of arsenic carcinogenesis: an integrated approach. Mutat. Res. 533, 37-65.

Rundle A., Tang D., Hibshoosh H., Schnabel F., Kelly A., Levine R., Zhou J., Link B. and Perera F. (2002). Molecular epidemiologic studies of polycyclic aromatic hydrocarbon-DNA adducts and breast cancer. Environ. Mol. Mutat. 39, 201-207.

Russo C., Rocco L., Morescalchi M. and Stingo V. (2004). Assessment of environmental stress by the micronucleus test and the Comet assay on the genome of teleost populations from two natural environments. Ecotox. Environ. Saf. 57:168-174.

Sakai T., Yanagihara S. and Ushio K. (1981). Erythrocyte factors concerned in the inhibition of ALA-D by lead. Br. J. Ind. Med. 38, 268-74.

Sakai T., Morita Y. and Takeuchi Y. (1996). Relationship between lead exposure and genetic polymorphism of delta-aminolevulinic acid dehydratase. Porphyrins 5, 233-236.

Sakai T. and Morita Y. (1996). Aminolevulinic acid in plasma or whole blood as a sensitive indicator of lead effects, and its relation to the other heme-related parameters. Int. Arch. Occup. Environ. Health 68, 126-132.

Sakai T. (2000). Biomarkers of lead exposure. Ind. Health 38, 127-142.

Sánchez-Chardi A., Peñarroja-Matutano C., Oliveira-Ribeiro C. and Nadal J. (2007). Bioaccumulation of metals and effects of a landfill in small mammals. Part II. The wood mouse, *Apodemus sylvaticus*. Chemosphere 70, 101-109.

Sánchez-Galán S., Linde A., Ayllon F. and García-Vázquez E. (2001). Induction of micronuclei in eel (*Anguilla anguilla* L.) by heavy metals. Ecotox. Environ. Saf. 49, 139-143.

Scheepers P. (2008). The use of biomarkers for improved retrospective exposure assessment in epidemiological studies: summary of an ECETOC workshop. Biomarkers 13, 734-748.

Schoen A., Beck B., Sharma R. and Dube E. (2004). Arsenic toxicity at low doses: epidemiological and mode of action considerations. Toxicol. Appl. Pharmacol. 198, 253- 267.

Schwartz B., Lee B., Stewart W., Ahn K., Springer K. and Kelsey K. (1995). Association of aminolevulinic acid dehydratase genotypes with plant exposure duration, and blood lead and zinc protopophyrin levels in Korean lead workers. Am. J. Epidemiol. 142, 738-745.

Sharma R. and Shupe J. (1977). Lead, cadmium, and arsenic residues in animal tissues in relation to those in their surrounding habitat. Sci. Total Environ. 7, 53-62.

Shi X., Jiang H., Mao Y., Ye J. and Saffiotti U. (1996). Vanadium(IV) mediated free radical generation and related 2'-deoxyguanosine hydroxylation and DNA damage. Toxicol. 106, 27-38.

Shore R. (1995). Predicting cadmium, lead and fluoride levels in small mammals from soil residues and by species-species extrapolation. Environ. Pollut. Ser. A. 88, 333-340.

Shugart R. (1988). Quantitation of chemically induced damage to DNA of aquatic organisms by alkaline unwinding assay. Aquat. Toxicol. 13, 43-52.

Shugart L. and Theodorakis C. (1994). Environmental genotoxicity: probing the underlying mechanisms. Environ. Health Perspect. 102, 13-17.

Silva J., Freitas T., Marinho R., Speit G. and Erdtmann B. (2000). Alkaline single-cell gel electrophoresis (comet) assay for environmental in vivo biomonitoring with native rodents. Genet. Mol. Biol. 23, 241-45.

Singh J., McLean J., Pritchard D., Montaser A. and Patierno S. (1998). Sensitive quantitation of chromium-DNA adducts by inductively coupled plasma mass spectrometry with a direct injection high-efficiency nebulizer. Toxicol. Sci. 46, 260-265.

Singh N., McCoy M., Tice R. and Schneider L. (1988). A simple technique for quantification of low levels of DNA damage in individual cells. Exp. Cell Res. 175, 184-191.

Singh R. and Farmer P. (2006). Liquid chromatography electrospray ionization mass spectrometry: the future of DNA adduct detection. Carcinogenesis. 27, 178-196.

Sivikova K. and Dianovsky J. (1995). Sister-chromatid exchanges after exposure to metal-containing emissions. Mutat. Res. 327, 17-22.

Smith E. and Cairns J. (1993). Extrapolation methods for setting ecological standards for water quality: statistical and ecological concerns. Ecotoxicology. 2, 203-219.

Snape J., Maund S., Pickford D. and Hutchinson T. (2004). Ecotoxicogenomics: the challenge of integrating genomics into aquatic and terrestrial ecotoxicology. Aquat. Toxicol. 67, 143-154.

Sorensen M., Autrup H., Moller H., Hertel O., Jensen S., Vinzents P., Knudsen L. and Loft S. (2003). Linking exposure to environmental pollutants with biological effects. Mutat. Res. 544, 255-271.

Strom M., Ramsdell S. and Archuleta S. (2002). Aminolevulinic acid dehydratase activity in American dippers (*Cinclus mexicanus*) from a metal-impacted stream. Environ. Toxicol. Chem. 21, 115-120.

Stýblo M., Drobná Z., Jaspers I., Lin S. and Thomas D. (2002). The role of biomethylation in toxicity and carcinogenicity of arsenic: a research update. Environ. Health Perspect. 110, 767-771.

Swenberg J., Fryar-Tita E., Jeong Y., Boysen G., Starr T., Walker V. and Albertini R. (2008). Biomarkers in toxicology and risk assessment: informing critical dose-response relationships. Chem. Res. Toxicol. 21, 253-265.

Tapisso J., Marques C., Mathias M. and Ramalhinho L. (2009). Induction of micronuclei and sister chromatid exchange in bone-marrow cells and abnormalities in sperm of Algerian mice (*Mus spretus*) exposed to cadmium, lead and zinc. Mutat. Res. 678, 59-64.

Tekin D., Kayaalt Z. and Söylemezoglu T. (2012). The effects of metallothionein 2A polymorphism on lead metabolism: are pregnant women with a heterozygote genotype for metallothionein 2A polymorphism and their newborns at risk of having higher blood lead levels?. Int. Arch. Occ. Environ. Health 85, 631-637.

Thompson L. (2005). Unraveling the Fanconi anemia-DNA repair connection. Nat. Genet. 37, 921-922.

Theodorakis C., Swartz C., Rogers W., Bickham J., Donnelly K., Marshall S. and Adams S. (2000). Relationship between genotoxicity, mutagenicity, and fish community structure in a contaminated stream. J. Aquat. Ecosys. Stress Recovery. 7, 131-143.

Tice R., Agurell E., Anderson D., Burlinson E., Hartmann A., Kobayashi H., Miyamae Y., Rojas E., Ryu J.C. and Sasaki F. (2000). Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ. Mol. Mutagen. 35, 206-221.

Timbrell J. (1998). Biomarkers in toxicology. Toxicology 129, 1-12.

Todd A., Wetmur J., Moline J., Godbold G., Levin S. and Landrigan P. (1996). Unraveling the chronic toxicity of lead: an essential priority for environmental health. Environ. Health Perspect. 4, 20-29.

Torres K. and Johonson M. (2001). Bioaccumulation of metals in plants, arthropods, and mice at a seasonal wetland. Environ. Toxicol. Chem. 20, 2617-2626.

Tovar-Sánchez E., Cervantes T., Martínez C., Rojas E., Valverde M., Ortiz-Hernández L. and Mussali-Galante P. (2012). Comparison of two wild rodent species as sentinels of environmental contamination by mine tailings. Env. Sc. Poll. Res. 19, 1677-1686.

Tremblay A., Lesbarreres D., Merritt T. and Wilson C. (2008). Genetic structure and phenotypic plasticity of yellow perch (*Perca flavescens*) populations influences by habitat, predation and contamination gradients. Integr. Environ. Assess Manag. 4, 264-266.

Tucker J. and Preston J. (1996). Chromosome aberrations, micronuclei, aneuploidy, sister chromatid exchanges, and cancer risk assessment. Mutat. Res. 365, 147-159.

Tugwood J., Hollins L. and Cockerill M. (2003). Genomics and the search for novel biomarkers in toxicology. Biomarkers 8, 79-92.

Ungherese G., Mengoni A., Somigli S., Baroni D., Focardi S. and Ugolini A. (2010). Relationship between heavy metals pollution and genetic diversity in Mediterranean population of the sandhopper *Talitrus saltator* (Montagu) (Crustaceae, Amphipoda). Environ. Pollut. 158, 1638-1643.

Valavanidis A., Vlahoyianni T. and Fiotakis K. (2005). Comparative study of the formation of oxidative DNA damage marker 8-hydroxy-2-deoxygua-nosine (8-OHdG) adduct from the nucleoside 2-deoxyguanosine by transition metals and suspensions of particulate matter in relation to metal content and redox reactivity. Free Radical Res. 39, 1071-1081.

Valavanidis A., Vlachogianni T. and Fiotakis C. (2009). 8-hydroxy-2-deoxyguanosine (8-OHdG): a critical biomarker of oxidative stress and carcinogenesis. J. Environ. Sci. Health Part C. 27, 120-139.

Valavanidis A. and Vlachogianni T. (2010). Metal pollution in ecosystems. Ecotoxicology studies and risk assessment in the marine environment. Sci. Adv. Environ. Toxicol. Ecot. Issues. chem-tox-ecotox.org/wp/wp-content/uploads/2010/01/02-Metals-17_01_2010.pdf [on line].

Valko M., Morris H. and Cronin M. (2005). Metals, toxicity and oxidative stress. Curr. Medicinal Chem. 12, 1161-1208.

Valko M., Rhodes C., Moncol J., Izakovic M. and Mazura M. (2006). Free radicals, metals and antioxidants in oxidative stress-induced cancer. Chem-Biol Interact. 160, 1-40.

Valverde M. and Rojas E. (2009). Environmental and occupational biomonitoring using the comet assay. Mutat Res. 681, 93-109.

Van der Oost R., Beyer J. and Vermeulen N. (2003). Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ. Toxicol. Pharmacol. 13, 57-149.

Vanden-Heuvel J. and Davis J. (1999). Molecular approaches to identify exposure and risk to specific environmental pollutants. Biomarkers 4, 93-105.

Vanparys C., Dauwe T., Campenhout K., Bervoets L., Coen W., Blust R. and Eens M. (2008). Metallothioneins and aminolevulinic acid dehydratase (ALAd) as biomarkers of metal pollution in great tits (*Parus major*) along a pollution gradient. Sci. Total Environ. 401, 184-193.

Venier P., De Pittà, C., Pallavicini, A., Marsano, F., Varotto, L., Romualdi C., Dondero F., Viarengo A. and Lanfranchi G. (2006). Development of mussel mRNA profiling: can gene expression trends reveal coastal water pollution? Mutat. Res. 602, 121-134.

Viarengo A., Burlando B., Dondero F., Marro A. and Fabbri R. (1999). Metallothionein as a tool in biomonitoring programmes. Biomarkers 4, 455-466.

Wang T., Yang Hsu T., Chung C., Wang A., Bau D. and Jan K. (2001). Arsenite induces oxidative DNA adducts and DNA-protein cross-links in mammalian cells. Free Radical Biol. Med. 31, 321-330.

Wang A. and Fowler B. (2008). Roles of biomarkers in evaluating interactions among mixtures of lead, cadmium and arsenic. Toxicol. Appl. Pharmacol. 233, 92-99.

Wang R., Bencic D., Biales A., Lattier D., Kostich M., Villenueve D., Ankley A., Lazorchak J. and Toth G. (2008). DNA microarray-based ecotoxicological biomarker discovery in a small fish model species. Environ. Toxicol. Chem. 27, 664-675.

Warchałowska-Śliwa E., Niklińska M., Görlich G., Michailova P. and Pyza E. (2005). Heavy metal accumulation, heat shock protein expression and cytogenetic changes in *Tetrix tenuicornis* (L.) (Tetrigidae, Orthoptera) from polluted areas. Environ. Pollut. 133, 373-381.

Watson W. and Mutti A. (2004). Role of biomarkers in monitoring exposures to chemicals: present position, future prospects. Biomarkers 9, 211-242.

Wilson D. and Thompson L. (2007). Molecular mechanisms of sister-chromatid exchange. Mutat. Res. 616, 11-23.

Wong R., Kuo C., Hsu M., Wang T., Chang P., Wu T. and Huang S. (2005). Increased levels of 8-hydroxy-2-deoxyguanosine attributable to carcinogenic metal exposure among schoolchildren. Environ. Health Perspect. 113, 1386-1390.

Yauk C. and Quinn J. (1996). Multilocus DNA fingerprinting reveals high rate of heritable genetic mutation in herring gulls nesting in an industrialized urban site. Proc. Natl. Acad. Sci. 93, 12137-12141.

Zhitkovich A. (2005). Importance of chromium−DNA adducts in mutagenicity and toxicity of chromium(VI). Chem. Res. Toxicol. 18, 3-11.

APÉNDICE C

Environ Sci Pollut Res (2012) 19:1677–1686
DOI 10.1007/s11356-011-0680-4

RESEARCH ARTICLE

Comparison of two wild rodent species as sentinels of environmental contamination by mine tailings

E. Tovar-Sánchez · L. T. Cervantes · C. Martínez · E. Rojas · M. Valverde · M. L. Ortiz-Hernández · P. Mussali-Galante

Received: 19 July 2011 / Accepted: 22 November 2011 / Published online: 9 December 2011

Abstract
Background Contamination with heavy metals is among the most hazardous environmental concerns caused by mining activity. A valuable tool for monitoring these effects is the use of sentinel organisms. Particularly, small mammals living inside mine tailings are an excellent study system because their analysis represents a realistic approach of mixtures and concentrations of metal exposure.
Purpose We analyzed metal tissue concentrations and DNA damage levels for comparison between genders of a sentinel (*Peromyscus melanophrys*) and a nonsentinel (*Baiomys musculus*) species. Also, the relationship between DNA damage and the distance from the contamination source was evaluated.
Methods This study was conducted in an abandoned mine tailing at Morelos, Mexico. Thirty-six individuals from both species at the exposed and reference sites were sampled. Metal concentrations in bone and liver of both species were analyzed by atomic absorption spectrophotometry, and DNA damage levels were assayed using the alkaline comet assay.
Results In general, concentrations of zinc, nickel, iron, and manganese were statistically higher in exposed individuals. A significant effect of the organ and the site on all metal tissue concentrations was detected. Significant DNA damage levels were registered in the exposed group, being higher in *B. musculus*. Females registered higher DNA damage levels than males. A negative relationship between distance from the mine tailing and DNA damage in *B. musculus* was observed.
Conclusions We consider that *B. musculus* is a suitable species to assess environmental quality, especially for bioaccumulable pollutants—such as metals—and recommend that it may be considered as a sentinel species.

Keywords *Baiomys musculus* · *Peromyscus melanophrys* · Heavy metals · DNA damage · Comet assay

Responsible editor: Elena Maestri

E. Tovar-Sánchez · L. T. Cervantes · C. Martínez
Departamento de Sistemática y Evolución, Centro de Investigación en Biodiversidad y Conservación, Universidad Autónoma del Estado de Morelos,
Av. Universidad 1001, Col. Chamilpa,
Cuernavaca, Morelos CP. 62209, Mexico

E. Rojas · M. Valverde · P. Mussali-Galante (✉)
Departamento de Medicina Genómica y Toxicología Ambiental, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México,
México D.F. CP 04510, Mexico
e-mail: pmussali@yahoo.com.mx

M. L. Ortiz-Hernández
Centro de Investigaciones en Biotecnología, Universidad Autónoma del Estado de Morelos,
Av. Universidad 1001, Col. Chamilpa,
Cuernavaca, Morelos CP. 62209, Mexico

1 Introduction

Mining activity is known to pollute natural resources in many parts of the world. Due to runoff, leaching, and aerial drift, these materials are present in surface, spring and drinking water, sediments, soil, and air, often in concentrations well above the permissible levels (Erry et al. 2000; Pascoe et al. 1994). Hence, exposure to contaminants through these environmental pollutants is common and represents a major problem of ecosystem health, with effects at all levels of biological organization.

Numerous areas have been polluted by metals and metalloids from mining activities, especially from waste discharges and an inappropriate management of mine tailings. Many

mines are abandoned once the mine has been exhausted (Phelps and McBee 2008), and generally little or no effort is done to minimize potential environmental effects. There are numerous such sites along the world, and Mexico is not an exception. Particularly, in Huautla (Morelos, Mexico), a mining district known for its historic mining activity since the sixteenth century until 1988, especially lead, silver, and zinc. There were four mines in the region, none of which are active today, but tons of toxic wastes have been discharged in the area. It is estimated that there are about 780 thousand tons of mine wastes, and the majority of them rich in Pb, Mn, and Cd that have not been processed or neutralized. It is estimated that the main contaminants of soil and groundwaters are arsenic and lead (Volke et al. 2004, 2005).

Many studies have shown that responses to toxic stress in different species and among genders vary widely. Therefore, the search for new sentinel organisms that may serve as model organisms to infer and predict effects in other levels of biological organization becomes important. Also, use of biomonitors, native species that indicate environmental quality, has increased due to the realization that many laboratory models exhibit weak correlations with natural ecosystems (Batty et al. 1990; Levengood and Heske 2008; Phelps and McBee 2008; Smith et al. 2002). In this context, wild animals, especially rodents living inside mine tailings, are an excellent model to study these exposures since they are in close contact with soil, water, and air pollutants and their analysis represent a realistic approach of mixtures and concentrations of metal exposure.

The genus *Peromyscus* has been widely studied in ecotoxicological surveys, especially in superfund sites (Laurinolli and Bendell-Young 1996; Levengood and Heske 2008; Phelps and McBee 2008; Talmage and Walton 1991) due to specific life history traits such as short life spans, close contact with contaminants due to ingestion and inhalation of contaminated soils, small home range sizes, easy capture, wide geographical distribution, and are typically found in both contaminated and noncontaminated sites. These traits make them suitable candidates as sentinel organisms. Other rodents with the same characteristics that inhabit inside mine tailing but have not been studied in ecotoxicological terms and as sentinel organisms are individuals of the genus *Baiomys*.

The use of biomarkers of early effect to detect more sensitive species that may serve as sentinel organisms is suggested. In this context levels of DNA damage are a reliable tool to estimate effects of contaminants in wild populations and the consequences in other levels of biological organization. In this regard, the alkaline single-cell gel electrophoresis or "comet assay" is a sensitive, reliable method for detecting alkali-labile and delayed repair sites measured as DNA single-strand breaks (SSB) in eukaryotic individual cells. The comet assay has been considered as an early biomarker of effect, widely used to asses DNA damage in studies on environmental exposure to genotoxins, such as metals (Frenzilli et al. 2009; Mussali-Galante et al. 2005; Rojas et al. 1999; Valverde et al. 1997). Hence, the aims of this study were: (a) to evaluate mining toxic stress in a sentinel species (*Peromyscus melanophrys*) and a nonsentinel species (*Baiomys musculus*) that cohabit in an impacted metal mining site, (b) to analyze bone and liver metals concentration and DNA damage levels between *P. melanophrys* and *B. musculus* individuals, and (c) to assess the relationship between DNA damage and the distance from the contamination source.

2 Methods

2.1 Study sites

Reference site The town of Ajuchitlán is also located inside the "Reserva de la Biosfera Sierra de Huautla" (REBIOSH). It is located southwest of the municipality of Tlaquiltenango (18°27′ N–98°58′ W) in an altitude of 1.060 m (INEGI 2001, 2004; Fig. 1).

The reference site was chosen because there are no mines in or near this area and there are no records of any possible contamination by metals in the zone. Also, ecological and geographical characteristics are very similar to the exposed site (Martínez-Pacheco 2008; Mussali-Galante 2008). Moreover, we avoided agricultural activity at the sampling sites in order to evade any agrochemical contamination as a confounding factor. Ajuchitlán is located at 6.5 linear km from the exposed site.

Exposed site This study was conducted in Huautla, Mexico. This zone is located at the southern part of Morelos state in the municipality of Tlaquiltenango (18°25′ N–99°01′ W). Huautla is located inside a protected natural reserve known as the REBIOSH (Dorado et al. 2005; Volke et al. 2004; Werre and Ortiz-Hernández 2000). This zone was also decreed as "Patrimony of the humanity" by the UNESCO in 1996. As a result of mining activity, there is one mine tailing (18°26′ N–99°01′ W, 995 m a.s.l.) approximately at 500 m from Huautla town (Mussali-Galante 2008; INEGI 2009; Volke et al. 2004, Fig. 1). These residues were left in open air and near lakes that flow into the Amacuzac river. Hence, there are possibilities that during rainy season, these residues may lixiviate, leach, or run off to other zones (Volke et al. 2004). The mine tailing, which is surrounded by deciduous forest (Rzedowski 2006), has a pH of 8.20, a cation exchange capacity of 30.1 cmol(+)/kg, it contains high Pb and As concentrations (2,298 and 139 mg/kg, respectively), and the predominant particulate size is <45 μm (44.2%), being this fraction where the highest metal concentrations are contained [As (31.9%), Cd (26.0%), Pb (30.7%), and V (29.1%)] (Volke et al. 2004, 2005).

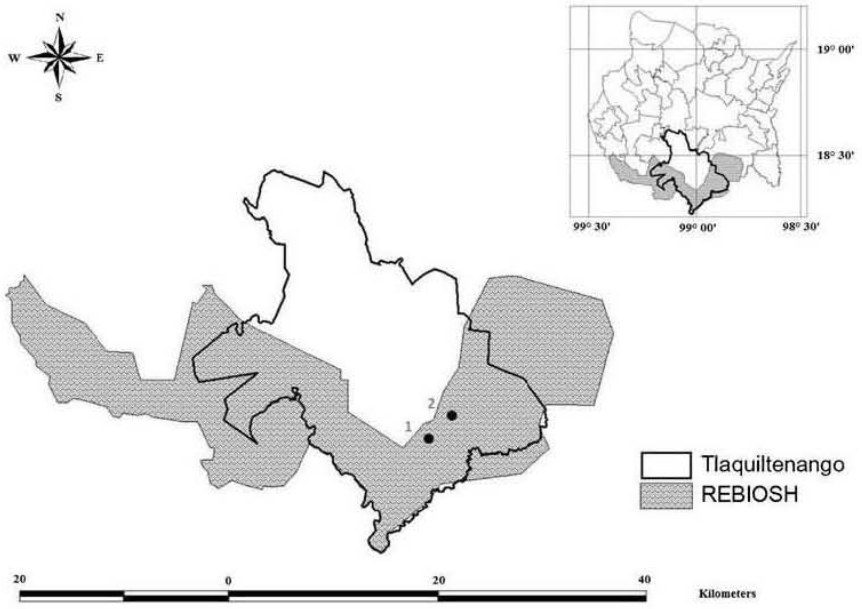

Fig. 1 Geographical distribution of the two study sites at the Reserva de la Biosfera Sierra de Huautla, Morelos, Mexico: control site (*1*), exposed site (*2*)

Study species B. musculus (Merriam 1892) is considered as endemic to Mexico; it distributes from southern Nayarit in Mexico to northern of Nicaragua. In Mexico it is present in deciduous forests and template regions. For Morelos state, *B. musculus* is the most abundant roden: species of the deciduous forest (Rivas 2006). Population densities vary from 15 to 20 individuals/ha, and their activity area is of 30 m of diameter, although these parameters vary between sites (Chavez and Espinosa 2005). In Morelos state, these small mammals are mice with a total longitude between 104 and 120 mm for females and from 105 to 120 for males, being the tale smaller than the body. Body weight oscillates between 8 and 13 g for females and 8 to 10 for males (Sánchez and Romero 1992). *B. musculus* is a generalist herbivorous mouse (Chávez y Espinosa 2005; Rivas 2006).

P. melanophrys (Coues 1874) is considered as endemic to Mexico; it occurs from Durango, Coahuila, Nuevo León and Tamaulipas to Chiapas state in Mexico. It is present in deciduous forests, being more common in arid zones than in template mountain regions (Sánchez and Romero 1992). It distributes all across Morelos state, occurring from 100 to 2,600 m a.s.l. (Musser and Carleton 2005), female registered a total longitude between 220 and 275 mm and male between 233 and 270 mm. Female adult individual weights oscillate between 30 and 58 g, and male 30 and 49 g. These small mammals are nocturnal and mostly herbivorous (Sánchez and Romero 1992).

2.2 Animal sampling

In the exposed site, four permanent grids were established (400 m each) for each orientation (east, west, south, and north). We established as the starting point the center of the mine tailing (18°26′04″ N 99°01′21″ W). In each grid, eight trap stations were established (32 in total) separated between them by 50 m. In each trap station, ten Sherman live traps were put (in total, 320 Sherman traps) with 50-m intervals along the grid in a perpendicular direction towards the permanent grid. The same sampling method was followed for the reference site, and the starting point was at 18°26′07″ N and 98°59′42″ W. All the procedures were done in accordance to the Mexican regulation about sampling and ethical handling of animal specimens FAUT-0251.

Sherman live traps were baited with oatmeal balls and peanut butter. Traps were set each evening (1800 hours) and checked in the next morning (0630 hours). Sampling was done from June to September 2009, until sampling 18 individuals for each species in each study site. Animals were transported live to the laboratory. Once in the laboratory, whole blood samples were taken from the sinus orbital with a sterilized and heparinized capillar tube and placed in sterile microcentrifuge tubes for the comet assay. Thereafter, mice were anesthetized with sodium phenobarbital (e.g., injection, 1 ml/kg), sexed and weighed, and necropsied immediately. Livers were removed and placed in acid-washed microcentrifuge tubes, respectively. Liver samples were stored at −80°C until metal

analyses. Finally, mice were put in the mastozoological collection at the "Centro de Investigación en Biodiversidad y Conservación," at the Autonomous University of Morelos State MOR-MAM-177-0705.

2.3 Metal analysis

Liver and bone tissue samples were dried to constant weight and subjected to acid digestion using a Microwave Accelerated Reaction System (CEM® MARS-5) with a 4:1 mixture of HNO_3 65% and HCl 37% (Baker) in closed Teflon bombs. The sample was solubilized and dissolved in distilled water and filtered; this solution was diluted to a final volume of 50 ml until analysis. A sample without tissue was processed simultaneously which was used as a control. The metals were analyzed by atomic absorption spectrophotometry (980 AA, GBC), with background correction. To ensure a satisfactory accuracy of the analysis, Standard Reference Material of National Institute and Technology and internal reference materials were used for precision, quality assurance and control for selected metal measurements. For each measurement, average values of three replicates were recorded. The recoveries of metals were within the range of 95.7% to 103%. The standards of the different elements were obtained from ULTRA Scientific. Calibration curves of different metals derived from these standards, proved to be a first-order reaction. Detection limits were 0.001 mg/l for Zn, 0.005 mg/l for Ni, 0.02 mg/l for Fe, and 0.002 mg/l for Mn.

2.4 Cell viability

The dual cell-stain assay described by Hartman and Speit (1997) was employed to determine the viability of the whole blood cells before the comet assay. In this assay, the integrity of the nuclear membrane and the metabolic activity of the lysosome are evaluated. The analysis was performed with a fluorescence microscope (Olympus BX60); four fields and at least 400 cells per slide were scored. The results were expressed as percentage of cells alive relative to control.

2.5 Alkaline comet assay

Alkaline single-cell gel electrophoresis assay was performed as described previously (Méndez-Gómez et al. 2008). An appropriate number of lymphocytes (≈500,000) were obtained and mixed with 75 μl of low melting point agarose (LMPA) 0.5%. The mixture was dropped on a slide precoated with 150 μl of 0.5% agarose, immediately covered with cover glass, and placed in a chilled steel tray for 1 min. The over glass was removed, and 75 μl of LMPA was applied. Slides were immersed in a chilled lysing solution pH 10 (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris; pH 10 fresh 10% dimethyl sulfoxide, and 1% Triton X-100). After lysis (4°C for at least 1 h), alkaline electrophoresis was performed. After electrophoresis, slides were gently removed and alkaline pH was neutralized with 0.4 M Tris, pH 7.5. Slides were dehydrated by two 10-min periods with 100% ethanol. Ethidium bromide (75 μl of a 20-μg/ml solution) was added to each slide, and a cover slip was placed on the gel. Individual cells were visualized at ×200 magnifications on an Olympus BX-60 microscope (Japan) with fluorescence attachments (excitation filter 515–560 nm and barrier filter 590 nm), and the extent of migration (tail-length value) was measured with a scaled ocular. For identifying tail image, the head of the comet was defined as the more brilliant circular region in the image. To evaluate DNA migration, 100 cells per individual were scored.

2.6 Data analyses

Three-factor ANOVAs were conducted (model I fixed effects, Zar 2010) to determine the effect of the rodent species (*B. musculus* and *P. melanophrys*), organ (bone and liver), site (control and exposed), and interaction organ × site on metal tissue concentrations (Zn, Ni, Fe, and Mn). Two-factor ANOVAs were conducted (model I fixed effects, Zar 2010) to determine the effect of the study site (control and exposed), gender (male and female), and interaction site × gender on tissue metal concentrations (Zn, Ni, Fe, and Mn) in *B. musculus* and *P. melanophrys*. Also, Tukey's analysis was conducted to determine significant differences for each mean metal concentration among genders and sites of each species (Zar 2010).

Nested ANOVAs were conducted (model I fixed effects, Zar 2010) to determine the effects of the species (*B. musculus* vs. *P. melanophrys*), of the site (exposed vs. control), of the gender (male vs. female), and of the individual on lymphocyte DNA damage (microns). Individuals were considered as a random factor nested within species, because they were representative of each population.

A Tukey's analysis was conducted to determine significant differences in mean damage among individuals of each species (Zar 2010). Finally, the regression analysis was used to test the relationship between mine tailing distance and DNA damage in lymphocytes of *P. melanophrys* and *B. musculus*. Data were log-transformed ($X'=\log X+1$). The software used for statistical analysis was STATISTICA 6.0 (Statsoft 1998).

3 Results

3.1 Metal tissue concentration analyses

Metal tissue concentrations (Zn, Ni, Fe, and Mn) in bone and liver did not vary significantly between rodent species

(*B. musculus* and *P. melanophrys*); it is worth noting that other metals such as Pb and Cu ranging from N.D. (not detectable levels) to 6.81 and N.D. to 100.55 μg g^{-1}, respectively, were also found but due to the great variability found, we decided not include them in the analysis. On the contrary, significant dependence of the organ (bone and liver) and the site (control and exposed) for all metal tissue concentrations was detected. In general, we did not observe a significant effect of the interactions on metal tissue concentrations, except for RS×O, RS×S, and O×S for Zn and O×S for Ni and Fe concentrations (Table 1).

In general, metal bone and liver concentrations in *B. musculus* and *P. melanophrys* were always significantly higher in the exposed site than in the control site, except for bone Mn concentrations in *P. melanophrys* and for liver Ni in both species. Females of both species, from the exposed site had always significantly higher Zn levels in both organs compared to males from the same site. This same pattern was also observed for bone Fe concentration in *B. musculus* and for bone and liver Mn concentrations in *P. melanophrys* (Tables 2 and 3).

3.2 Cellular viability

Cellular viability of peripheral blood lymphocytes of *B. musculus* and *P. melanophrys* was higher than 95% for the control group (mean±e.e.; 97.33±1.36%) and for the exposed group (95.5±0.84%).

3.3 DNA damage

In general, a significant effect of the site on DNA damage levels (SSB) was recorded ($F_{1,\ 2,000}$=7556.55, P<0.001), being higher for the exposed group (mean±e.e.; 167.88±0.69) than for the control group (41.35±0.30). Also, a significant effect of the rodent species on DNA damage levels was observed ($F_{1,\ 2,000}$=41.43, P<0.001), being greater for *B. musculus* (111.31±0.13) in comparison to *P. melanophrys* (97.84±0.12). A similar effect of the gender on DNA damage levels was registered ($F_{2,\ 2,000}$=132.62, P<0.001), being higher in females (118.67±1.36) than in males (90.56±1.36). A significant effect of the individual on DNA damage levels was also observed ($F_{16,\ 2,000}$=118.58, P<0.001).

In general, female individuals of *B. musculus* showed significant higher levels of DNA damage in comparison with male individuals, independently of the study site (control vs. exposed). The same pattern was registered for exposed group of *P. melanophrys*, however in the control site an inverse pattern was observed (Figs. 2 and 3).

A significant effect of the interaction species×site on DNA damage levels was registered ($F_{1,\ 2,000}$=8.20, P<0.01). Individuals from the control site registered the lowest DNA damage levels for both species (*P. melanophrys* 36.08±0.29, *B. musculus* 46.56±0.50) in comparison with the exposed site (*P. melanophrys* 159.60±0.71, *B. musculus* 176.07±1.15). Moreover, *B. musculus* populations registered the highest DNA damage levels in both sites.

A significant effect of the gender × site on the mean DNA damage levels was recorded ($F_{2,\ 2,000}$=714.70, P<0.001). Individuals from the control site registered the lowest DNA damage levels for both genders (males 39.96±0.45, females 42.74±0.41) in comparison with the exposed site (males 141.16±0.49, females 194.59±0.95). It is worth noting that females from both sites showed the highest levels of DNA damage.

A significant effect of the interaction species × gender on DNA damage levels was registered (F_1=1994.61, P<0.001). *B. musculus* females (136.47±2.04) presented higher levels of DNA damage than *P. melanophrys* females (100.69±1.70). Meanwhile, males from both species registered an inverse pattern (*P. melanophrys*, 95.00±1.38; *B. musculus*, 86.16±1.59).

3.4 Mine tailing distance

Lineal regression analysis detected a negative and significant relationship between distance (in meters) from the mine

Table 1 Three-factor ANOVA results to determine the effects of rodent species *B. musculus* and *P. melanophrys*, organ (bone and liver), site (control and exposed), and interactions on metal tissue concentrations (Zn, Ni, Fe, and Mn)

Source	Zn $F_{1,\ 72}$	Ni $F_{1,\ 72}$	Fe $F_{1,\ 72}$	Mn $F_{1,\ 72}$
Rodent species (RS)	0.08, ns	0.00, ns	0.91, ns	1.25, ns
Organ (O)	78.50*	32.59*	43.05*	21.54*
Site (S)	108.48*	14.64*	37.60*	6.43*
RS × O	4.73**	0.01, ns	0.01, ns	2.72, ns
RS × S	6.59***	0.58, ns	0.71, ns	0.15, ns
O × S	25.40*	14.41*	5.62**	3.33, ns
RS × O × S	0.45, ns	0.62, ns	0.46, ns	0.04, ns
Error				

ns no significant differences
*P<0.001; **P<0.05; ***P<0.01

Table 2 Mean metal concentrations (in micrograms/gram dry weight) and standard deviation in the bone of *B. musculus* and *P. melanophrys* from the control and exposed sites

Site/gender	*N*	Zn Metal concentration ($\mu g\ g^{-1}$)±SD	Ni Metal concentration ($\mu g\ g^{-1}$)±SD	Fe Metal concentration ($\mu g\ g^{-1}$)±SD	Mn Metal concentration ($\mu g\ g^{-1}$)±SD
		B. musculus			
Control (male)	5	22.65±4.29a	6.26±1.74a	17.01±7.80a	1.47±0.62a
Control (female)	5	29.83±6.23a	6.98±3.76a	16.24±7.29a	0.91±0.72a
$Control_{total}$	10	26.24±6.19A	6.62±2.65A	16.63±6.77A	1.19±0.68A
Exposed (male)	5	104.33±29.41b	55.20±26.12b	22.24±14.09a	3.74±2.53a
Exposed (female)	5	150.17±36.19c	46.45±30.54b	92.67±42.03b	2.82±2.07a
$Exposed_{total}$	10	130.52±39.29B	50.20±34.66B	62.49±48.65B	3.21±2.22B
ANOVA	Site (S)	$F_{1,\ 16}$=50.89*	$F_{1,\ 16}$=12.87**	$F_{1,\ 16}$=8.10***	$F_{1,\ 16}$=6.52***
	Gender (G)	$F_{1,\ 16}$=4.51***	$F_{1,\ 16}$=0.19, ns	$F_{1,\ 16}$=5.90***	$F_{1,\ 16}$=1.29, ns
	S×G	$F_{1,\ 16}$=1.86, ns	$F_{1,\ 16}$=0.24, ns	$F_{1,\ 16}$=6.16***	$F_{1,\ 16}$=0.29, ns
		P. melanophrys			
Control (male)	5	60.52±4.29a	12.92±5.59a	23.26±10.95a	1.92±1.01a
Control (female)	5	56.10±6.23a	13.91±6.47a	18.53±9.13a	3.07±1.30a
$Control_{total}$	10	58.06±6.19A	13.47±5.74A	20.64±14.04A	2.56±1.52A
Exposed (male)	5	96.37±29.41a	43.18±6.19b	36.28±6.06ab	1.87±1.40a
Exposed (female)	5	139.72±36.19b	41.43±12.63b	53.79±7.28b	6.32±1.67b
$Exposed_{total}$	10	118.54±39.29B	42.30±8.94B	40.03±31.41B	4.10±2.90A
ANOVA	Site (S)	$F_{1,\ 16}$=7.69**	$F_{1,\ 16}$=4.35***	$F_{1,\ 16}$=11.81**	$F_{1,\ 16}$=0.93, ns
	Gender (G)	$F_{1,\ 16}$=4.55***	$F_{1,\ 16}$=0.01, ns	$F_{1,\ 16}$=0.80, ns	$F_{1,\ 16}$=5.17***
	S×G	$F_{1,\ 16}$=0.54, ns	$F_{1,\ 16}$=0.02, ns	$F_{1,\ 16}$=0.84, ns	$F_{1,\ 16}$=1.11, ns

Two-factor ANOVA results to determine the effect of study sites (S), gender (G), and for the interaction S × G on tissue metal concentrations are given. Different letters denote significant differences at $P<0.05$ (Turkey's honestly significant difference test)

ns no significant differences

*$P<0.001$; **$P<0.01$; ***$P<0.05$

tailing and DNA damage levels in *B. musculus* individuals, for both genders, females ($r=-0.93$, $r^2=0.987$, $P<0.05$) and males ($r=-0.95$, $r^2=0.91$, $P<0.05$; Fig. 4). This is indicative for a heavy metal concentration gradient. Lineal regression analysis do not detected a significant relationship between distance from the mine tailing and DNA damage levels in male ($r=-0.30$, $r^2=0.09$, $P>0.05$) and female ($r=-0.16$, $r^2=0.03$, $P>0.05$) individuals of *P. melanophrys*.

4 Discussion

Metals are among the most prevalent common and toxic substances released into the environment that have a profound effect on living organisms (Bickham et al. 2000; Dimsoski and Toth 2001; Guttman 1994; Moore et al. 2004; Mussali-Galante and Fortoul 2008; Theodorakis 2001; Tremblay et al. 2008). The analysis of DNA alterations in organisms has been shown to be a highly suitable method for evaluating the genotoxic contamination of environments, being able to detect exposure to low concentrations of contaminants in a wide range of species. In general, these methods have the advantage of detecting and quantifying the genotoxic impact without requiring a detailed knowledge of the identity and the physical/chemical properties of the contaminants present. In this respect we studied the genetic damage in two rodent species, *P. melanophrys*, a sentinel species, and *B. musculus*, a nonsentinel species, that cohabit in an impacted metal mining site.

A great increase of DNA damage was found in both species, being more marked in *B. musculus* than in *P. melanophrys*; this is in agreement with several previous reports showing metals genotoxic potential (Park and Park 2011; Pra et al. 2008; Scheirs et al. 2006). We also observed a gender effect, where females had more DNA damage levels than males. At this respect, gender differences in response to metal insults have been found in many species (Pra et al. 2008). One of the reasons that may account for the differences observed is a hormonal effect, perhaps due to higher levels of xenobiotic metabolism, which may be the result of the effects of female sex hormones (Mugford and Kedderis 1998). The same authors comment that sex-specific differences can

Table 3 Mean metal concentrations (micrograms/gram dry weight) and standard deviation in the liver of *B. musculus* and *P. melanophrys* from the control and the exposed sites

Site/gender	N	Zn Metal concentration ($\mu g\ g^{-1}$)±SD	Ni Metal concentration ($\mu g\ g^{-1}$)±SD	Fe Metal concentration ($\mu g\ g^{-1}$)±SD	Mn Metal concentration ($\mu g\ g^{-1}$)±SD
		B. musculus			
Control (male)	5	20.29±2.00a	0.88±0.37a	57.19±9.40a	0.78±0.10a
Control (female)	5	21.33±3.21a	0.97±0.3 a	58.72±4.96a	0.81±0.13a
$Control_{total}$	10	20.82±2.24A	0.93±0.32A	57.95±6.78A	0.80±0.11A
Exposed (male)	5	47.75±3.01b	0.76±0.58a	172.12±69.12ab	1.21±0.21b
Exposed (female)	5	74.98±18.70c	1.11±0.54a	94.16±18.93b	1.11±0.16ab
$Exposed_{total}$	10	61.36±19.13B	0.94±0.54A	133.14±62.27B	1.16±0.17B
ANOVA	Site (S)	$F_{1,16}$=53.23*	$F_{1,16}$=0.01, ns	$F_{1,16}$=12.92**	$F_{1,16}$=16.25**
	Gender (G)	$F_{1,16}$=6.47***	$F_{1,16}$=0.67, ns	$F_{1,16}$=5.34***	$F_{1,16}$=0.15, ns
	S × G	$F_{1,16}$=5.55***	$F_{1,16}$=0.23, ns	$F_{1,16}$=3.61, ns	$F_{1,16}$=0.46, ns
		P. melanophrys			
Control (male)	5	19.90±4.16a	0.99±0.17a	46.87±4.89a	0.50±0.09a
Control (female)	5	21.83±3.49a	1.00±0.08a	57.19±3.71a	0.67±0.09a
$Control_{total}$	10	20.87±3.60A	0.99±0.12A	52.03±6.86A	0.65±0.19A
Exposed (male)	5	33.44±5.44b	1.10±0.27a	112.30±19.87b	0.63±0.31a
Exposed (female)	5	57.13±7.18c	1.44±0.83a	136.52±24.89b	1.21±0.19b
$Exposed_{total}$	10	40.29±9.41B	1.27±0.58A	124.41±31.93B	0.97±0.35B
ANOVA	Site (S)	$F_{1,16}$=40.91**	$F_{1,16}$=1.47, ns	$F_{1,16}$=29.28*	$F_{1,16}$=5.89***
	Gender (G)	$F_{1,16}$=6.62***	$F_{1,16}$=0.45, ns	$F_{1,16}$=1.67, ns	$F_{1,16}$=7.99***
	S × G	$F_{1,16}$=3.75, ns	$F_{1,16}$=0.43, ns	$F_{1,16}$=0.30, ns	$F_{1,16}$=1.80, ns

Two-factor ANOVA results to determine the effect of study sites (S), gender (G), and for the interaction S×G on tissue metal concentrations are given. Different letters denote significant differences at $P<0.05$ (Tukey's honestly significant difference test)

ns no significant differences

*$P<0.001$; **$P<0.01$; ***$P<0.05$

produce lower clearances of chemicals in the sex with the slower rate of metabolism, resulting in prolonged half-lives and higher blood concentrations, which may cause toxicity. Conversely, if a toxic metabolite is produced by metabolism, the sex with the lower metabolic activity may be less susceptible to the specific chemical-induced toxicity. In addition, the sex-dependent variation in metabolism by rodents may be the result of differential evolution of isoforms of cytochromes P450 in mammals (Mugford and Kedderis 1998). However, other causes could be involved such as different metal intake and accumulation, in our case seems to be more important metal intake than a hormonal effect, because *B. musculus* females have more Zn and Fe than males and also more than *P. melanophrys* females. This could reflect increase susceptibility in *B. musculus* females (Table 2). Also, Zn tends to increase in soft tissues in the females of some mammals such as the mole (Komarnicki 2000). More specifically, steroid hormone receptors are Zn finger proteins and Zn is also part of several enzymes, including some related to antioxidant systems such as cytosolic superoxide dismutase (Lopes et al. 2002). On the whole, the gender differences of essential elements like Fe and Zn may be associated with differences in the metabolic profile of metals involved in the activity of sexual hormones, the intake or uptake of metals, nutritional requirements, or interactions between elements (Sánchez-Chardi et al. 2009).

Despite the genetic damage found in both species, we only observed differences in the relationship between the induction of DNA damage and the distance to the mine tailing in *B. musculus* individuals. This observation could be due to the restricted home range of *B. musculus* animals in comparison with a greater home range of *P. melanophrys* (Chavez and Espinosa 2005).

For an animal to be considered a key sentinel species, it must satisfy certain requirements. Many animal groups have been hailed as valuable sentinels, including birds (Furness and Nettleship 1991; George 1999; Pastor et al. 2004), marine mammals (Colborn and Smolen 1996; Wells et al. 2004), aquatic organisms (O'Conner 2002; Zelikoff 1998), and domestic pets (Bukowski and Wartemberg 1997; National Research Council 1991). Terrestrial mammalian

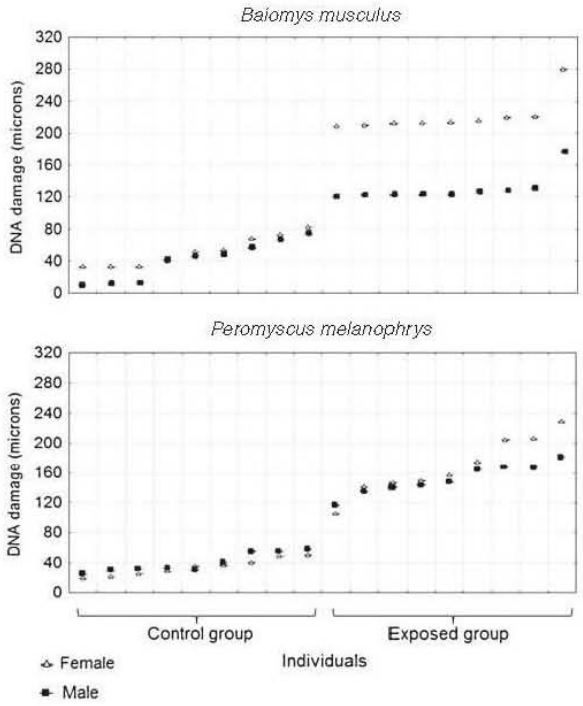

Fig. 2 Mean±standard deviation of DNA single-strand breaks of each male and female individual of *B. musculus* and *P. melanophrys* in Huautla, Morelos, Mexico

wildlife, in particular, possesses multiple characteristics that are favorable to their inclusion in studies concerning environmental and human health (O'Brien et al. 1993). Mammalian wildlife has physiological systems that are similar to those of humans in mediating the uptake, distribution, metabolism, and elimination of toxicants. Furthermore, humans and many species of mammalian wildlife inhabit

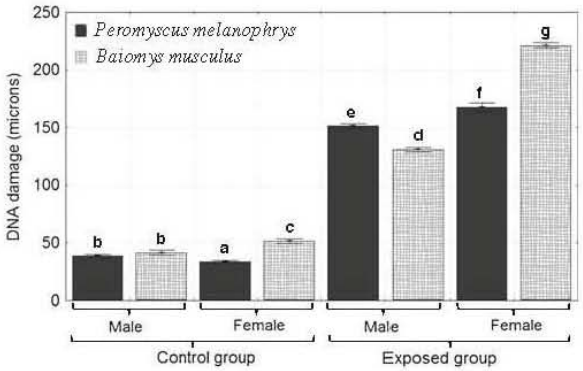

Fig. 3 Mean±standard deviation of DNA single-strand breaks of each male and female individual of *B. musculus* and *P. melanophrys* of the control and exposed group in Huautla, Morelos, Mexico. *Different letters* show significant differences at $P<0.05$ (Tukey's honestly significant difference test)

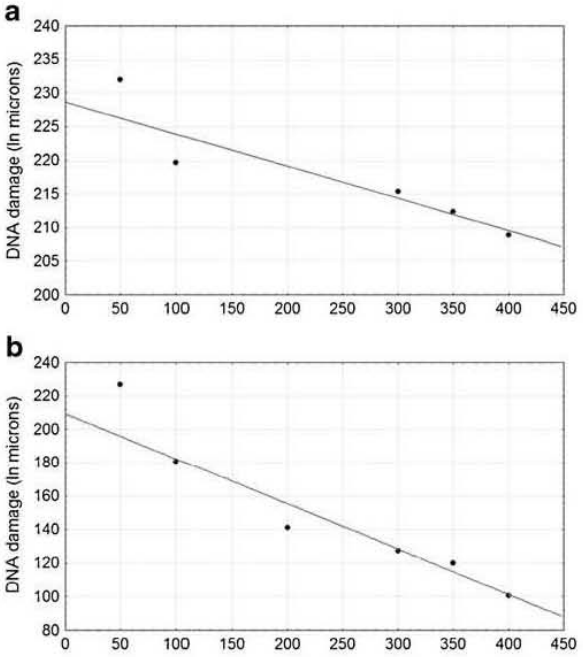

Fig. 4 Relationships between mine tailing distance and the induction of DNA single-strand breaks for **a** female and **b** male individuals of *B. musculus* in Huautla, Morelos, Mexico

similar ecosystems and are exposed to common climates, food sources, and pollutants. Free ranging wildlife can integrate ecological factors and real world complexities (e.g., stresses associated with disease, human disturbance, or temperature) that are not attainable in controlled laboratory bioassays. As such, the toxicological information derived from mammalian wildlife may have more applicability to humans than data collected from non-mammalian models, such as fish, reptiles, and birds (Basu et al. 2007).

Sentinel species provide a useful tool for following heavy-metal exposure over time as well as serving as an early warning. The value of sentinel species depends partly on how metals accumulate in the organisms, and the amounts that cause effects. A useful organism must be sufficiently sensitive to the effects of a metal to provide early warning, yet not so sensitive that it reflects changes that are not biologically meaningful, both for the organism and for species that consume it. In addition, sentinels should be relatively easy to monitor over long periods of time and large spatial areas, and such monitoring should be economically feasible (Peakall 1992).

In our study, *B. musculus* individuals behaved as sentinel species, reflecting metal bioaccumulation, DNA damage as a result of metal exposure, clear gender differences, and a relationship between mine tailing distance and DNA damage level was established. Moreover, *B. musculus* is a species with a well-known biology and widespread distribution. We

consider that *B. musculus* is a suitable species to assess environmental quality, especially for bioaccumulable pollutants—such as metals—and recommend that it may be considered as a sentinel species. Also, our findings highlight the long-lasting effects of metals even long periods of time after the close of the mine.

Acknowledgments The authors thank Edith Rivas, M. Mora Jarvio, Evodio Rendon Alquicira, and G. Rangel Altamirano for the technical assistance. This research was supported by Programa de Mejoramiento al Profesorado scholarship to L.T.C.R. and Consejo Nacional de Ciencia y Tecnología scholarship to C.M.B.

References

Basu N, Scheuhammer AM, Bursian SJ, Elliot J, Rouvinen-Watt K, Man Chan H (2007) Mink as a sentinel species in environmental health. Environ Res 103:130–144

Batty J, Leavitt R, Biondo N, Polin D (1990) An ecotoxicological study of a population of white-footed mouse inhabiting a polychlorinated biphenyls-contaminated area. Arch Environ Contam Toxicol 19:283–290

Bickham J, Sandhu S, Hebert P, Chikhi L, Athwal R (2000) Effects of chemical contaminants on genetic diversity in natural populations: implications for biomonitoring and ecotoxicology. Mutat Res 463:33–51

Bukowski JA, Wartemberg D (1997) An alternative approach for investigating the carcinogenicity of indoor air pollution: pets as sentinels of environmental cancer risk. Environ Health Perspect 105:1312–1319

Chavez C, Espinosa L (2005) *Baiomys musculus* (Merriam, 1892). In: Ceballos G, Oliva G (eds) Los mamíferos silvestres de México. Comisión Nacional para el Conocimiento y Uso de la Biodiversidad, Fondo de Cultura Económica, Mexico, pp 667–668

Colborn T, Smolen MJ (1996) Epidemiological analysis of persistent organochlorine contaminants in cetaceans. Rev Environ Contam Toxicol 146:91–172

Coues E (1874) Synopsis of the Muridae in North America. Proc Acad Nat Sci Phila 3:173–196

Dimsoski P, Toth G (2001) Development of DNA-based microsatellite marker technology for studies of genetic diversity in stressor impacted populations. Ecotoxicology 10:229–232

Dorado O, Maldonado B, Arias D, Sorani V, Ramírez R, Leyva E (2005) Programa de conservación y manejo Reserva de la Biosfera Sierra de Huautla. Comisión Nacional de Áreas Naturales Protegidas, Mexico

Erry BV, Macnair MR, Meharg AA, Shore RF (2000) Arsenic contamination in wood mice (*Apodemus sylvaticus*) and bank voles (*Clethrionomys glareolus*) on abandoned mine sites in southwest Britain. Environ Pollut 110:179–187

Frenzilli G, Nigro M, Lyons BP (2009) The comet assay for the evaluation of genotoxic impact in aquatic environments. Mutat Res 681:80–92

Furness RW, Nettleship DN (1991) Seabirds as monitors of changing marine environments. Int Ornithol Congr 20:2237–2279

George JC (1999) Birds as bioindicators of ecotoxicological effects of heavy metal pollution. J Anim Morphol Physiol 46:1–22

Guttman SI (1994) Population genetic structure and ecotoxicology. Environ Health Perspect 102:97–100

Hartman A, Speit G (1997) The contribution of cytotoxicity to DNA effects in the single cell gel test (comet assay). Toxicol Lett 90:183–188

INEGI (2001) Conteo Nacional de Población y Vivienda, Mexico

INEGI (2004) Información Geográfica del Estado de Morelos, Mexico

INEGI (2009) Información Geográfica del Estado de Morelos, Mexico

Komarnicki GJ (2000) Tissue, sex and age specific accumulation of heavy metals (Zn, Cu, Pb, Cd) by populations of the mole (*Talpa europaea* L.) in a central urban area. Chemosphere 41:1593–1602

Laurinolli M, Bendell-Young LI (1996) Copper, zinc, and cadmium concentrations in *Peromyscus maniculatus* sampled near an abandoned copper mine. Arch Environ Contam Toxicol 30:481–486

Levengood J, Heske E (2008) Heavy metal exposure, reproductive activity and demographic patterns in white-footed mice (*Peromyscus leucopus*) inhabiting a contaminated floodplain wetland. Sci Total Environ 389:320–328

Lopes PA, Viegas-Crespo AM, Nunes AC, Pinheiro T, Marques C, Santos MC, Mathias ML (2002) Influence of age, sex, and sexual activity on trace element levels and antioxidant enzyme activities in field mice (*Apodemus sylvaticus*) and (*Mus spretus*). Biol Trace Elem Res 85:227–239

Martínez-Pacheco M (2008) Evaluación de los efectos genotóxicos de metales presentes en el agua de bebida de la población de Hauautla, Morelos. Dissertation. Universidad Nacional Autónoma de México

Méndez-Gómez J, García-Vargas GG, López-Carrillo L, Calderón-Aranda ES, Gómez A, Vera E, Valverde M, Cebrián ME, Rojas E (2008) Genotoxic effects of environmental exposure to arsenic and lead on children in region Lagunera, Mexico. Ann N Y Acad Sci 1140:358–367

Merriam C (1892) Description of nine new mammals collected by E. W. Nelson in the states of Colima and Jalisco, Mexico. Proc Biol Soc Wash 7:164–174

Moore M, Depledge M, Readman J, Leonard D (2004) An integrated biomarker-based strategy for ecotoxicological evaluation of risk in environmental management. Mutat Res 552:247–268

Mugford CA, Kedderis GL (1998) Sex dependent metabolism of xenobiotics. Drug Metab Rev 30:441–498

Mussali-Galante P (2008) Estudio sobre la inducción de daño al ADN en sangre periférica de individuos expuestos a metales en al agua de bebida, en la población de Huautla, Morelos. M.Sc. thesis. Universidad Nacional Autónoma de México

Mussali-Galante P, Fortoul T (2008) Atmospheric pollution. In: Maes F (ed) Environmental research progress, Nova Science Publishing, pp 147–160

Mussali-Galante P, Ávila M, Piñón G, Martínez G, Rodríguez V, Rojas M, Ávila M, Fortoul TI (2005) DNA damage as an early biomarker of effect in human health. Toxicol Ind Health 21:155–166

Musser GG, Carleton MD (2005) Superfamily Muroidea. In: Wilson DE, Reeder DM (eds) Mammal species of the world a taxonomic and geographic reference. Johns Hopkins University Press, Baltimore, pp 894–1531

National Research Council (1991) Animals as sentinels of environmental health hazards. Committee on Animals as Monitors of Environmental Hazards. National Academy Press, Washington

O'Brien DJ, Kaneene JB, Poppenga RH (1993) The use of mammals as sentinels for human exposure to toxic contaminants in the environment. Environ Health Perspect 99:351–368

O'Conner TP (2002) National distribution of chemical concentrations of mussels and oysters in the USA. Mar Environ Res 53:117–143

Park JH, Park E (2011) Influence of iron-overload on DNA damage and its repair in human leukocytes in vitro. Mutat Res 718:56–61

Pascoe G, Blanchet R, Linder G (1994) Bioavailability of metals and arsenic to small mammals at a mining waste-contaminated wetland. Arch Environ Contam Toxicol 27:44–50

Pastor N, Baos R, López-Lázaro M, Jovani R, Tella J, Hajji N, Hiraldo F, Cortés F (2004) A 4 year follow-up analysis of genotoxic damage in birds of the Doñana area (south west Spain) in the wake of the 1998 mining waste spill. Mutagenesis 19(1):61–65

Peakall D (1992) Animal biomarkers as pollution indicators. Chapman and Hall, London

Phelps K, McBee K (2008) Ecological characteristics of small mammal communities at a superfund site. Am Midl Nat 1:57–68

Pra D, Rech-Frenke SI, Giulian R, Yoneama ML, Ferraz-Diaz J, Erdtmann B, Pegas-Henriques JA (2008) Genotoxicity and mutagenicity of iron and copper in mice. Biometals 21:289–297

Rivas E (2006) Díeta de *Baiomys musculus* (Merriam, 1982) en la Sierra de Huautla y su relación con la perturbación y la estacionalidad. Dissertation. Universidad Autónoma de Estado de Morelos, Mexico

Rojas E, López MC, Valverde M (1999) Single cell gel electrophoresis assay: methodology and applications. J Chromatogr 722:225–254

Rzedowski J (2006) Vegetación de México. Fondo de Cultura Económica, Mexico

Sánchez CH, Romero MLA (1992) Mastofauna silvestre del ejeido el Limón, municipio de Tepalcingo, Morelos. Universidad Ciencia y Tecnol 2:87–95

Sánchez-Chardi A, Oliveira-Ribeiro C, Nadal J (2009) Metals in liver and kidneys and the effects of chronic exposure to pyrite mine pollution in the shrew *Crocidura russula* inhabiting the protected wetland of Doñana. Chemosphere 76:387–394

Scheirs J, Coan A, Covaci A, Beernaert J, Kayawe M, Caturla M, Wolf H, Baert P, Van-Oostveldt P, Verhagen R, Blust R, Coen W (2006) Genotoxicity in wood mice (*Apodemus sylvaticus*) along a pollution gradient: exposure age, and gender-related effects. Environ Toxicol Chem 25:2154–2162

Smith P, Cobb G, Harper F, Adair B, McMurry S (2002) Comparison of white-footed mice and rice rats as biomonitors of polychlorinated biphenyl and metal contamination. Environ Pollut 119:261–268

Statsoft (1998) STATISTICA. For Windows. Manual version 6.0. Statsoft, Oklahoma

Talmage S, Walton B (1991) Small mammals as monitors of environmental contaminants. Rev Environ Contam Toxicol 119:47–145

Theodorakis CW (2001) Integration of genotoxic and population genetic endpoints in biomonitoring and risk assessment. Ecotoxicology 10:245–256

Tremblay A, Lesbarreres D, Merritt T, Wilson C (2008) Genetic structure and phenotypic plasticity of yellow perch (*Perca flavescens*) populations influences by habitat, predation, and contamination gradients. Integr Environ Assess Manag 4:264–266

Valverde M, López MC, López I, Sánchez I, Fortoul T, Ostrosky-Wegman P, Rojas E (1997) DNA damage in leukocytes and bucal and nasal epithelial cells of individuals exposed to air pollution in Mexico city. Environ Mol Mutagen 30:147–152

Volke ST, Velasco TA, De la Rosa PA, Solórzano OG (2004) Evaluación de tecnologías de remediación para suelos contaminados con metales. Etapa I. Secretaría de Medio Ambiente y Recursos Naturales, Mexico

Volke ST, Velasco TA, De la Rosa PA, Solórzano OG (2005) Evaluación de tecnologías de remediación para suelos contaminados con metales. Etapa II. Secretaría de Medio Ambiente y Recursos Naturales, Mexico

Wells RS, Rhinehart WL, Hansen LJ, Sweeney JC, Townsend FI, Stone R, Casper DR, Scott MD, Hohn AA, Rowles TK (2004) Bottlenose dolphins as marine ecosystem sentinels: developing a Elath monitoring system. Ecohealth 1:246–254

Werre F, Ortiz-Hernández L (2000) Monografía geologica-minera del estado de Morelos. Consejo de Recursos Minerales, Mexico

Zar J (2010) Biostatistical analysis. Prentice-Hall, New-Jersey

Zelikoff JT (1998) Biomarkers of immunotoxicity in fish and other non-mammalian sentinel species: predictive value for mammals? Toxicology 129:63–71

APÉNDICE D

Genetic Structure and Diversity of Animal Populations Exposed to Metal Pollution

Patricia Mussali-Galante[1], Efraín Tovar-Sánchez [2], Mahara Valverde [1] and Emilio Rojas [1].

[1] Departamento de Medicina Genómica y Toxicología Ambiental, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, México, D.F. C.P. 04510.[2] Departamento de Sistemática y Evolución, Centro de Investigación en Biodiversidad y Conservación, Universidad Autónoma del Estado de Morelos, Av. Universidad 1001, Col. Chamilpa, Cuernavaca, Morelos, CP 62210, México.

Corresponding author: Rojas E. Departamento de Medicina Genómica y Toxicología Ambiental, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, México, D.F. C.P. 04510; E-mail address: emilior@servidor.unam.mx

Contents

1 Introduction

The introduction of toxic substances into the environment by anthropogenic or natural activities is widespread and causes significant perturbation. Therefore, increasing attention has been focused on better understanding the long-term ecological effects of chronically exposed populations, communities and ecosystems. The increased understanding of such effects has not only resulted from enhanced biomonitoring activities, but also from developing new toxicity and ecotoxicity data for various species.

Genetic change in exposed populations is one of the more subtle effects of environmental exposures, and has potentially large, long-term effects (Van Straalen and Timmermans 2002). Thus, there are benefits for monitoring the genetic patterns of wild populations for assessing environmental impacts in natural systems (Belfiore and Anderson 1998; Medina et al. 2007).

Metals are among the most common substances released into the environment, and these compounds can have a profound effect on living organisms (Guttman 1994; Bickham et al. 2000; EPA 2000; Dimsoski and Toth 2001; Theodorakis 2001; Moore et al. 2004; WHO 2007; Tremblay et al. 2008). This is under-scored by the fact that metal-induced effects are among the top ten concerns of the U.S. Environmental Protection Agency (EPA, 2000). Metals affect natural populations in many ways. Genetic changes are one of the most important alterations that may occur, and when they occur they may disrupt the genetic equilibrium at all levels of biological organization.

Genetic structure represents the rearrangement of allelic and genotypic frequencies of populations and represents how genetic variation is distributed within and among populations. Evolution and the maintenance of genetic structure in space and time are dependent on natural

selection forces, genetic drift, mating systems, recombination, mutations and gene flow (Loveless and Hamrick 1984; Coutellec and Barata 2011). In addition to these evolutionary forces, how genetic variation is distributed within and among populations is determined by exterior factors, such as ecological phenomena, particularly habitat disturbance and fragmentation, and life history traits of the species (Barret and Khon 1991). Ecological disturbances may be of natural (e.g., geologic processes, volcanic eruptions, and fires) or anthropogenic (e.g., agricultural practices, mining and other industrial activities) origin.

Genetic ecotoxicology is the study of xenobiotic-induced changes in the genetic material of natural biota. Direct alterations to genes and gene expression may occur from exposures, or the pollutants may induce selective effects on gene frequencies (Anderson et al. 1994). In this context, contaminant-induced selection and genetic bottlenecks are mechanisms by which the genetic structure of populations can become altered. Both factors may affect the adaptive ability of a contaminant-exposed population and may have consequences at the community and ecosystem levels (Gillespie and Guttman 1989; Theodorakis et al. 2000; Harper-Arabie et al. 2004; Athrey et al. 2007; Brown et al. 2009). Consequently, changes in diversity and genetic structure parameters may be used as bioindicators of ecosystem health, which is defined as a comprehensive, multiscale, dynamic, hierarchical measure of system resilience, organization and vigor (Ehrenfeld 1992).

Although numerous studies address the ecotoxicity of metals, few have addressed the topic of genetic ecotoxicity. Among studies that have focused on environmentally-stressed populations and their genetic population-level responses, two principal approaches have been utilized. In the first approach, the genetic or molecular non-neutral markers are identified that are linked to resistance or sensitivity to environmental stressors, or a combination of both stressors, in select

species. In the second approach, changes in genetic diversity parameters are addressed in the exposed populations, by using neutral molecular markers, such as allozymes, mitochondrial DNA analyses, RAPDs (random amplified polymorphic DNA), SSRs (single sequence repeats) or microsatellites and AFLPs (amplified fragment length polymorphic DNA) (D´Surney et al. 2001; Hoffman and Dabron 2007).

In this review, we endeavor to summarize the key work that has been performed to assess the effects of metals on the genetic pattern of several organisms; these effects are often a result of metal-induced environmental stress on natural animal populations. In genetic ecotoxicology, it is important to recognize the differences between genetic changes due to genotoxic or mutagenic mechanisms of action, genetic alterations due to ecological processes, such as genetic drift and bottlenecks, and environmental changes that alter genetic variability in natural populations in terms of allele frequencies, heterozygosity levels and gene flow. Additionally, we address the potential relationship between exposure to chemical agents and changes in genetic structure, and the possible long-term consequences of chronically exposed populations. Another issue that we address is the use of sentinel species adequate to study genetic ecotoxicological questions.

Finally, we reach conclusions and make suggestions on what is required to strengthen this area of research, and we also propose a new class of biomarkers, termed “biomarkers of permanent effect”. These biomarkers are useful tools to estimate ecosystem health through the evaluation of changes in structure and genetic diversity of the exposed populations.

2 Implications of Metal Toxicity on Genetic Populations

Metals are among the most toxic elements to nearly all living organisms (EPA 2000; WHO 2007). The relationship between metal toxicity and a plethora of effects in living organisms is well established. Studies of populations exposed to metals in occupational or environmental settings were among the first to establish a quantitative relationship between effects and external exposure and/or internal dose received (Bernard 2008). The field of genetic toxicology is usually regarded as the study of the mechanisms of action of xenobiotics as regards their effects on DNA. The goal of research in this area is to assess the risks posed to individuals by xenobiotics capable of inducing adverse health effects. For many years, studies in this field were focused on the effects of acute exposures to single toxicants at high doses. However, in genetic ecotoxicology, threats to populations and communities arising from chronic exposures to mixtures of chemical agents at lower doses (realistic exposures) are also of potential concern (Depledge 1994). Thus, establishing links between the molecular and cellular effects of metals and their possible consequences at higher levels of biological organization becomes truly necessary when attempting to understand population level responses to chemicals, such as metals. Bickham and Smolen (1994) defined the term "emergent effects". The research results they report explains that although the damage from xenobiotic exposure is at the cellular or subcellular levels, emergent effects are observed at higher levels of biological organization. However, the effects produced are not predictable by merely knowing the mechanism of action of the chemical agent in question. Therefore, these higher-level effects can be assessed in wild animal populations only by using ecological indicators, such as shifts in the genetic pools of populations chronically exposed to environmental pollutants.

Because genetic variation is the basis for biodiversity and evolution (Duan et al. 2001; Medina et al. 2007), and because the loss of genetic variability may be permanent (depending on the

population size and mutation rates), investigating how chemicals exert their effects on the genetic pool in exposed populations (changes in genetic structure) is a priority in environmental biomonitoring and conservation programs.

Various authors (Van Straalen 1999; Van Straalen and Timmermans 2002; Maes et al. 2005) have described ways in which chemical agents may alter the genetic variability of an exposed population, to wit: 1) toxicants can be genotoxic (i.e., they directly or indirectly alter the DNA molecule) or mutagenic (i.e., they increase mutation rates). Genotoxic substances can affect different DNA repair processes by interacting with the key enzymes responsible for DNA damage repair, and thereby increase mutation rates; 2) toxicants may favor more tolerant genotypes and/or eliminate intolerant genotypes, changing the genetic composition of the exposed population towards a higher mean tolerance; 3) toxicants may cause bottlenecks that reduce the size of a population; and 4) toxicants may alter exchange of migrant individuals among populations.

Therefore, studying genetic ecotoxicology endpoints are as important as many other endpoints when being employed to predict the risks of populations exposed to pollutants.

3 Genotoxic Effects vs. Genetic Changes Caused by Natural Processes

Understanding how contaminants affect population genetic parameters may provide key insights about the consequences of exposure at the population level of the analyzed species. Therefore, studying the genotoxic effects of metals should become a routine and vital component of ecotoxicology, along with performing biomonitoring and ecological risk assessment (Theodorakis 2001; Benton et al. 2002; Gardeström et al. 2008).

To achieve this goal, it is important to delineate between genetic changes due to genotoxic exposure and genetic alterations as a result of: evolutionary forces, different mating systems, ecological factors or species life history traits, environmental changes that alter the genetic variability in natural populations in terms of allele frequencies, heterozygosity levels and gene flow.

DNA damage from, for example, the formation of DNA adducts, base pair modifications, DNA strand breaks and chromosome rearrangements are among the most common biomarkers of early adverse effects from toxic exposure. These insults may have serious consequences on the health of the population. If these alterations occur in somatic cells, a number of immediate effects may occur, such as cell death, or the accumulation of mutations and/or transformation into a malignant phenotype (Weinstein 1988). DNA damage in somatic cells can reduce the longevity of the individual (Agarwal and Sohal 1994), alter the age structure of the population (Theodorakis 2001), modify the size of the individual (individual size has implications for survival, fecundity, bioenergetics and behavior; any perturbation in size structure has the potential to induce effects at the population level or above) or alter the sex proportion of the population (there may be gender-related responses to DNA damage) (Scheirs et al. 2006).

Because the DNA molecule is the unit of inheritance, DNA alterations (mutation and translocation) in germ cells can be passed to the next generation, causing teratogenicity, low viability, low fertility, and low reproductive success. Higher mutation rates produce damaging effects and may lead to a decrease in the average fitness of the population (Anderson et al. 1994; Guttman 1994; Belfiore and Anderson 1998; Bickham et al. 2000; Yauk et al. 2000; Theodorakis 2001; Maes et al. 2005; Medina et al. 2007; Gardeström et al. 2008). Therefore, because genotoxic alterations in somatic cells are numerous and affect adults, they affect the current

population, whereas germ cell mutations from genotoxic stress, although less numerous may affect future generations and have long-term effects. Hence, alterations in both cell types potentially affect the genetic composition of populations in multiple ways (Bickham and Smolen 1994; Belfiore and Anderson 1998; Yauk et al. 2000; Bickham et al. 2008) (Fig. 1).

The use of population genetic tools to analyze the effects of contaminants on a species is difficult and requires an extraordinarily detailed experimental design. To discriminate between chemically-induced vs. natural process-induced alterations in the genetic composition of natural populations, many authors (Endler 1995; Belfiore and Anderson 1998; Baker et al. 2001; Staton et al. 2001) have made recommendations to enhance the robustness of experimental design. First, using multiple reference sites along with multiple experimental sites is recommended (Ross et al. 2002). Second, correlating observed genetic effects with biomarkers for internal doses (i.e., contaminant levels in organs or tissues) and external exposures (i.e., levels of contaminants in air, soil, and water) is suggested. Third, site sampling should be performed over time to establish the patterns resulting from non-contaminated factors, such as revolving ecological conditions and population cycles. To assess the relationships between contaminant exposure and changes in genetic patterns, gradient effects should be evaluated (De wolf et al. 2004; Bourret et al. 2008; Durrant et al. 2011). Additionally, the use of biomarkers to detect early effects, such as different types of DNA damage, which are indicative of exposure to genotoxic contaminants in somatic cells and germ cells, should be used to determine the relationship between DNA damage and population genetic responses (Belfiore and Anderson 1998; Theodorakis 2001; Benton et al. 2002; Ross et al. 2002; Moore et al. 2004). Moreover, biomarkers should be chosen that reflect changes in the fitness of an organism (e.g., premature death, ability to mate, fecundity, viability

of offspring, etc.) because these changes can have the greatest influence at the population level (Evenden and Depledge 1997).

Chen and Hebert (1999) suggested using molecular and phylogenetic tools to analyze the mutations via a technique termed ‘‘terminal branch haplotype analysis”. A mutation originating from natural processes or one that is chemically induced should exhibit a low frequency, and its nucleotide sequence should differ by only one base pair from its more common ancestral haplotype. The analysis of nucleotide sequences should identify any new haplotypes originating at the branch tips of the phylogenetic tree. The variants that branch more deeply within the tree or differ by more than a single base substitution from the closest related haplotype are most likely the result of gene flow from close populations. This method could prove useful for studies that use population genetic analysis as a toxicological investigation tool (Bickham et al. 2000; Theodorakis 2001; Theodorakis et al. 2001). In addition to obtaining information about the effects of chemical agents on genetic diversity, the foregoing approaches may provide additional information on how dispersal and migration patterns influence the biological effects caused by contamination (Theodorakis 2001; Eeva et al. 2006; Gardeström et al. 2008).

4 Population Genetic Responses to Environmental Metal Stress

Population genetic responses to chemical exposures, especially metals, can have a profound effect on the genetic variability of chronically exposed populations. These responses are driven in two general directions: increased genetic variation because of new mutations induced directly by the genotoxic agent(s), or decreased genetic variation resulting from population processes, such as bottlenecks or selection that will also alter allele and genotype frequencies in these

populations. In both cases, the changes in genetic variation may result from adaptation to polluted environments (Bickham et al. 2000; Berckmoes et al. 2005; Maes et al. 2005; Gardeström et al. 2008; Durrant et al. 2011).

Since the year 2000, 33 studies were reported in the Hermes, PubMed and Biological abstracts databases, in which the genetic structure and diversity of animal populations exposed to metals were analyzed. Of these studies, nine were review articles (Bickham et al. 2000; Clements 2000; Belfiore and Anderson 2001; Staton et al. 2001; Theodorakis 2001; Van Straalen and Timmermans 2002; Medina et al. 2007; Morgan et al. 2007; Hoffmann and Willi 2008), and 24 were original research reports. Among these original reports, 15 aquatic ecosystems and ten terrestrial ecosystems were examined.

In the aquatic ecosystem studies that were performed, 15 different species were analyzed, and a decrease in the genetic diversity of the exposed species was reported in ten of the publications. In contrast, no effects of metal pollution on the genetic diversity patterns of the exposed populations vs. the reference population were reported in four of the studies.

A decrease genetic diversity in the exposed species was reported for the majority of the studies (73.3%). In contrast, metal pollution had no effect on the genetic diversity patterns of the exposed populations vs. the reference population in four aquatic species (26.7%) (Table 1).

In the studies performed on terrestrial ecosystems, ten different species were analyzed, and decreased genetic diversity was reported for 40% of these exposed species. In addition, 40% of the exposed species exhibited increased genetic diversity, and genetic diversity was not affected by metal exposure in only two species (20%) (Table 1).

In the majority of the studies reviewed, mining activity or processes related to mining constituted the source of the contamination. The most common metals found in the aquatic and terrestrial ecosystems were Cd, Zn, Cu and Pb. However, the occurrence of these metals differed between the aquatic (Cd=Zn>Cu>Pb>Hg) and terrestrial (Cu>Cd>Pb>Zn>Ni) environments (Table 1).

Among the molecular markers used to assess the genetic diversity in the impacted populations, microsatellite markers were used (29.6%) most frequently, followed by allozyme electrophoretic techniques (25.9%), RAPD markers (18.5%), mitochondrial DNA analysis (14.8%) and other molecular markers (minisatellite mutations, electrophoretic analysis and AFLP) (11.1%) (Table 1).

In general, most of the aquatic ecosystem studies performed during the last decade disclosed that reduced genetic diversity occurred in the animal populations exposed to a single metal or a mixture of metals; however, this pattern becomes less clear when terrestrial ecosystems were analyzed (Table 1).

Among the reported studies, very different animal populations (from copepods to wild birds), environments (aquatic vs. terrestrial), exposure conditions (single or metal mixtures), types of metals and degrees of disturbance (intensity, duration of exposure, affected area and magnitude) were analyzed. Because the large number of variables makes comparisons difficult, each study compared results with its own geographical reference site(s). In this review, we did not find means to make valid comparisons among studies on genetic diversity parameters.

There are many explanations for what causes reduced genetic diversity in exposed populations. Genotypic selection may affect genetic change at the population level. In addition, changes in population size may produce genetic bottlenecks and possibly genetic drift of the population.

Finally, changes in the demographic patterns and reduced migration rates may reduce the genetic diversity (Van Straalen and Timmermans 2002). Therefore, ecological processes, such as bottlenecks resulting from the genotoxic effects of metals, the selection of tolerant and the elimination of intolerant genotypes, or the reduction in offspring that contribute to the next generation may lead to a decrease in the genetic variation within populations chronically exposed to polluted environments. Because genetic variability is the basis for adaptation by natural selection, it is generally accepted that the loss of genetic variability makes it more difficult for a population to adapt to future changes in the environment. The reduced variation can lead to an increased extinction rate (Anderson et al. 1994; Bickham et al. 2000; Tremblay et al. 2008) (Fig. 1).

The loss of genetic diversity in populations subjected to anthropogenic stress is referred to as "genetic erosion" and this may be a factor of concern in assessing the risk of toxic chemicals (Van Straalen and Timmermans 2002). Frattini et al. (2008) and Ungherese et al. (2010) validated the use of molecular markers in genetic studies to support the "genetic erosion hypothesis", by showing that metal contamination has negative influences on genetic diversity. In contrast, Eeva et al. (2006) reported increased genetic variation in populations impacted by metal pollution. The free-living insectivorous passerine (*P. major*) populations living near a smelter exhibited statistically higher nucleotide diversity than did a reference population in an unpolluted site, suggesting that high mutation rates occur in contaminated environments. Additionally, Peles et al. (2003) reported higher levels of heterozygosity in an exposed population compared with the reference population. The report showed that the percentage of earthworms (*Lumbricus rubellus*) in the highest heterozygosity class was four times higher in the exposed than in the reference population. Bourret et al. (2008) assessed the level of

heterozygosity, allelic richness, diversity and internal relatedness (IR), a measure of individual genetic diversity in yellow perch (*Perca flavescens*) populations. A negative correlation was observed between each of the genetic diversity parameters and the metal concentrations. In contrast, the levels of IR indicated that the more contaminated individuals were genetically more diverse than the less contaminated individuals in the contaminated and reference populations. These results suggest that the less inbred perch were more tolerant to metal contamination under certain circumstances. The authors explained that, under these circumstances, one would predict that individual fitness will increase with individual genetic diversity, and consequently, the selective pressures exerted by Cd contamination should favor the maintenance of higher genetic diversity within the contaminated populations.

Reports of increased genetic diversity in exposed populations support the hypothesis that the vast majority of mutations that negatively affect fitness are expected to be deleterious; an increased mutation rate inside a population will also increase its mutational load. Additionally, several field studies have demonstrated that mutations accumulate more rapidly in environments that are more polluted (Yauk and Quinn 1996; Clements 2000; Rogstad et al. 2003; Gardeström et al. 2008; Peles et al. 2008). Therefore, it is expected that populations that are chronically exposed to pollutants will likely experience a steady decrease in fitness due to an increasing mutational load, which ultimately has the potential to drive a population to extinction (Lynch et al. 1995).

Because individual fitness should increase with an individual's genetic diversity, another possible scenario is based on the assumption that selective pressures favor more genetically diverse populations. Thus, contaminated populations may contain higher levels of genetic diversity (Bourret et al. 2008).

When using different genetic diversity endpoints, variable results (e.g., increased or decreased genetic variability) can be explained in a variety of ways:

1) Differences in the response to environmental stress have been attributed to species susceptibility, and as stated earlier, different responses to stress among populations of a single species have been documented (Diamond et al. 1991; Lee et al. 1993; Eeva et al. 2006). Also, different species in the same polluted environment produce diverse results, which have been attributed to differences in species metabolism (Eeva et al. 2006).

2) Populations that belong to different ecosystems (terrestrial vs. aquatic) will exhibit different responses, mainly because the routes of exposure and the bioavailability of metals are different between the systems.

3) The use of different techniques to analyze the genetic parameters can produce different results. The majority of researchers have examined genetic variation using microsatellite markers and at allozyme loci. As stated previously, many microsatellite loci are considered to be one of the best molecular markers (Yauk and Quinn 1996; Athrey et al. 2007; Tremblay et al. 2008); their high mutation rates and high variability make them one of the most sensitive markers for analyzing genetic variability within and between populations exposed to different concentrations of genotoxins. Additionally, the quantification of the genetic variation at allozyme loci using electrophoretic techniques is the second most frequently used method. Most allozyme studies examine the impacts of heavy metals on allozyme diversity in aquatic organisms because of the extensive pollution of aquatic ecosystems with metals, and because there is evidence of many metals inhibiting or altering enzymatic activities (Nevo et al. 1983; Benton et al. 2002; Keane et al. 2005; Maes et al. 2005). The results from studies of mosquito fish (Chagnon and Guttman

1989; Diamond et al. 1989; Newman et al. 1989; Roark et al. 2001) and aquatic invertebrates (Nevo et al. 1978; Battaglia et al. 1980; Gillespie and Guttman 1989; Patarnello et al. 1991; Ma et al. 2000; Benton et al. 2002; Kim et al. 2003; Keane et al. 2005; Maes et al. 2005; Gardeström et al. 2008) suggest that genotypic frequencies at allozyme loci are affected by contaminant exposure, and that there is not a unique response pattern. Single metals and mixtures of metals may elicit different responses among the array of genotypes at a locus and among populations of a single species (Diamond et al. 1991; Lee et al. 1992). However, because the genetic pool of a population is constantly modified by natural processes, such as mutations, gene flow, genetic drift, and natural selection, the cause-effect relationships between genetic alterations measured using molecular markers and environmental stress are difficult to establish using organisms collected in the field (Medina et al. 2007). Thus, different techniques may yield different results.

4) The chemical agents under investigation can also affect the results. Heavy metals have numerous mechanisms of action. Their toxicological properties vary depending on the compound, concentration, route of exposure, type of exposure (mixtures or single agents) and metabolism. Thus, results may vary because of the metal or metal mixture analyzed. Moreover, it is important to consider that responses to metal stress may be influenced by other classes of chemical agents (e.g., polycyclic aromatic hydrocarbons) that may also occur in polluted environments. In such cases, reciprocal interactions, cascades and indirect mechanisms can enhance or suppress the expected responses (Benedetti et al. 2007).

A number of researchers, who published the papers outlined in this review, measured metal concentrations in soil or water but did not measure the internal dose of the metals in tissues or organs of the exposed individuals (Duan et al. 2001; Peles et al. 2003; Berckmoes et al. 2005; Haimi et al. 2006; Jordaens et al. 2006; Matson et al. 2006; Athrey et al. 2007; Gardeström et al.

2008; Durrant et al. 2011). The internal metal concentrations may not have been measured because the small body size of the organisms involved may have made such measurements difficult. In other studies, the internal dose, but not the external dose, was measured (Benton et al. 2002; Maes et al. 2005; Eeva et al. 2006; Bourret et al. 2008). However, both the internal dose and external doses were measured in several studies (Kim et al. 2003; Ungherese et al. 2010), although two of these studies do not specify the type of metals examined or the internal concentrations found (Ma et al. 2000; Yauk et al. 2000). Moreover, in the majority of the studies, the exposure conditions, such as the type and duration of the exposure, are not well characterized.

Despite these shortcomings, most authors report that metal-polluted environments affect the genetic structure of impacted animal populations. Bickham et al. (2000) suggested that the observed genetic effects are independent of the mechanism of action of the chemical agents involved. We think this assertion should be taken with caution, both because it is controversial and needs further analysis. Certainly, genetic structure effects may result from toxic exposures. However, it is yet to be established whether the accepted mechanism of toxic action of chemical agents are independent of observed genetic pattern effects in any given metal-exposed population. Hence, authors of future genetic ecotoxicological studies should better describe the chemical agents, and the exposure conditions (external and internal metal concentrations)

The majority of studies performed during the last decade that have assessed population genetic responses have demonstrated adverse effects. In particular, populations inhabiting environments with higher levels of contamination have exhibited lower genetic diversity and population differentiation, lower reproductive success, reduced adaptive potential and lower fitness. Therefore, it appears that there is a potential association between metal contamination and

changes in the genetic structure of exposed populations (Table 1). Unfortunately, there are only a limited numbers of studies in which the genetic diversity of terrestrial ecosystems impacted by metal pollution have been analyzed.

5 Genetic Markers for Assessing Genetic Variability in Environmentally Impacted Populations

The application of DNA sequencing and polymerase chain reaction-based technologies over the last 20 years has revolutionized the science of generating high throughput genetic markers (D´Surney et al. 2001).

Molecular markers are observable traits (their expression indicates the presence or absence of certain genes) that play an important role in estimating the genetic diversity among individuals by comparing the genotypes at a number of polymorphic loci (Arif and Khan 2009). A number of molecular markers have been applied to genetic ecotoxicological research, including nuclear and mitochondrial DNA analyses, such as allozymes, restriction fragment length polymorphisms (RFLPs), SSRs, RAPDs, the DNA sequencing of mtDNA, and AFLPs (Table 2).

One of the oldest techniques used to assess genetic variability in natural populations is to analyze the electrophoretic shifts in the charge characteristics of enzymes produced by amino acid substitutions, namely allozyme analysis. The majority of allozymes exhibit co-dominant inheritance and the variants are attributed to nucleotide substitutions that induce replacement of charged amino acids. This technique can detect one-third of amino acid substitutions. However, the generally low level of polymorphisms at allozyme loci often limits their resolving power for detecting population differences (Keane et al. 2005). Despite its limited resolution, allozyme

analysis remains the simplest and most rapid technique for surveying genetic diversity in single-copy nuclear genes (Bickham et al. 2000).

The RFLP method uses restriction enzymes to detect variations in the primary structure of DNA. The number of bases in the restriction site and the genome-based composition determine the number of restriction sites. RFLP probes are usually considered loci and alleles defined by a specific probe-enzyme combination (Lowe et al. 2004). These markers are co-dominant, and a major advantage of RFLP probes is that they make it possible to detect DNA and organelle DNA polymorphisms in total DNA extracts. In addition, RFLP results are highly repeatable, and large amounts of variation can be detected. However, the RFLP method requires large quantities of DNA and only a limited number of suitable nDNA markers are available. Moreover, the detection of RFLPs is expensive and time-consuming (Lowe et al. 2004).

SSRs are widely used to analyze for genetic structure and variability. SSRs are short tandem repeats of mono- to tetra-nucleotide repeats, which are assumed to be randomly distributed throughout the nuclear and mitochondrial genomes. The SSR method detects length variations that result from changes in the number of repeated units, and their mode of inheritance is co-dominant. Mutations in SSRs are high compared with other DNA markers. SSRs are regarded to be one of the best molecular markers (Yauk and Quinn 1996; Athrey et al. 2007; Tremblay et al. 2008) due to their high mutation rates and high variability, which make them sensitive markers for analyzing genetic variability within and between populations. Unfortunately, identifying SSRs is expensive and requires cloning and sequencing. Although SSR primer pairs appear to be species-specific, cross-species amplification has been demonstrated, although reduced variability has been observed.

RAPDs utilize single decamer oligonucleotide primers to amplify regions of the genome by polymerase chain reaction (PCR). RAPD primers contain a random sequence, are relatively short and many of them are used to sample the whole genome. Sites in the genome that are flanked by perfect or imperfect inverted repeats permit multiple annealing of the primers. The primer annealing sites occur throughout the genome, from single-copy DNA sequences to multiple-copy DNA sequences, and in coding and non-coding regions. RAPDs are cheap, simple to use, require no sequence information, and a large number of putative loci can be obtained when using them. However, there are numerous disadvantages associated with these molecular markers; RAPDs are dominant markers, meaning that they cannot distinguish heterozygotes from homozygotes at the phenotypic level, and their degree of reproducibility is low. Additionally, the primer structure, product competition, product homology, allelic variation, genome sampling and non-independence of the loci are examples of other weaknesses associated with this methodology (Lowe et al. 2004; Arif and Khan 2009). To overcome these disadvantages, modifications to the technique have been proposed, such as sequence characterized amplified regions (SCARs) and randomly amplified microsatellite polymorphisms (RAMPO) (Lowe et al. 2004).

One of the most powerful tools in modern molecular population genetics is the nucleotide sequence analysis of mitochondrial DNA (mtDNA) (Bickham et al. 2000). The mitochondrial protein-coding regions are regarded to be powerful markers for genetic diversity analysis. The most studied of the mitochondrial genes for genetic diversity analyses include cytochrome b (cyt b), NADH dehydrogenase subunit 5 and mitochondrial cytochrome oxidase I (COI). Additionally, the highly polymorphic non-coding region of mtDNA, termed the control region (CR or D-loop), has been used in genetic diversity analyses because of its role in the replication and transcription of mtDNA. The D-loop region exhibits higher variation levels than the protein-

coding regions because of the reduced functional constraints and the relaxed selection pressure. The advantages of the sequence approach include the ability to target different mitochondrial genes, thus selecting for targets with an appropriate evolutionary rate and the higher resolution obtained by revealing the nucleotide sequence. Moreover, an advantage of the PCR-RFLP analysis of mtDNA is that homo- and heterozygosity values and allele/genotype frequencies can be determined for the genetic loci analyzed (Bickham et al. 2000; D´Surney et al. 2001; Arif and Khan 2009).

AFLPs are multilocus markers that involve the selective amplification of a subset of restriction fragments generated by the digestion of DNA with restriction enzymes, followed by ligation to specific adapters. Similar to RAPDs, these markers are dominant, although co-dominant AFLP markers may be detected because of small insertions or deletions in the restriction fragments (Lowe et al. 2004; Arif and Khan 2009). Compared with RAPDs and SSRs, AFLP markers can generate ten times the number of potential markers per genome (D´Surney et al. 2001). The comparison of the results obtained using SSRs, mtDNA or AFLPs (Lucchini 2003) suggests that AFLPs could be very useful for evaluating genetic diversity. Because they are easily amplified in any species, AFLP markers may prove to be a valuable tool for estimating genetic diversity in animal populations.

All of the aforementioned molecular markers have applications in genetic ecotoxicology studies. Because none of the markers is ideal, marker choice should be based on the hypothesis that is being tested, the properties of the marker system, the organism under investigation and the resources that are available for the research project.

6 Use of Sentinel Organisms for Genetic Ecotoxicological Studies

An important step in establishing links between pollution effects and population level responses is the utilization of sentinel organisms or bioindicator species. Sentinel organisms are a set of taxa that can be utilized to survey locales for increased mutation stressors (Yauk and Quinn 1996).

A variety of organisms have been studied for their potential to be biological indicators of different forms of chemical pollution. Certain species are known to be highly sensitive, either in their physiological response to contaminants, or by their ability to accumulate metals in a dose-dependent manner. These organisms respond to the environmental stress caused by one or more pollutants by changing their morphology and/or metabolism, and the nature of such changes are observable and measurable. For bioindicators to be sensitive, it is often necessary that the xenobiotic of interest be accumulated (Markert et al. 1999).

In many cases, sentinel species are used to assess risk to species that may be closely related evolutionarily or may occupy a similar niche within an ecosystem. In general, many species of wild animals (especially aquatic organisms) have been used as sentinel organisms in ecotoxicological studies with metals. Examples of sentinel species include mosquitofish (*Gambusia affinis*) (Roark et al. 2001), many isopod species, copepods and gastropods (Ross et al. 2002; Storelli and Marcotrigiano 2005; Gardeström et al. 2008), earthworms (*Lumbricus rubellus*) (Peles et al. 2003), many nematode species (Ekschmitt and Korthals 2006), zebra mussels (*Dreissena polymorpha*) (Sues et al. 1997), garden snails (*Helix aspersa*) (Nedjoud et al. 2009), various species of sea birds (Burger and Gochfeld 2004), two crayfish species (*Austropotamobius pallipes* and *Pacifastacus leniusculus*) (Antón et al. 2000), and many species of prawns, mussels and oysters (Ma et al. 2000; Ross et al. 2000; Storelli and Marcotrigiano 2005).

Although they are key components of ecosystems and occupy a variety of niches, few small mammalian species have been used as sentinel organisms. Small mammals are however attractive sentinel organismal candidates, because they are important nutrient recyclers, influence plant and insect communities and serve as prey for numerous predators (Levengood and Heske 2008). Several adverse effects have been documented to occur in small mammals after chronic metal exposure. Among these are teratogenesis, genotoxic-related diseases, and reproductive alterations (Baranski 1987; Talmage and Walton 1991; Sunderberg and Okarsson 1992; Eisler 1997; Husby et al. 1999; Bisser et al. 2004).

Other factors that make small mammals ideal for studying pollution effects are their wide geographical distribution and abundance, the fact that adults remain established in the same localized area, they exhibit generalized food habits, short life spans, and high reproductive rates, and they are easily captured(Talmage and Walton 1991; Pascoe et al. 1994; Laurinolli et al. 2006; Levengood and Heske 2008). Moreover, small mammals play an important role in food chains and are considered intermediates for metal transfer to higher trophic levels (Talmage and Walton 1991; Levengood and Heske 2008). In addition, mammals accumulate metals in different tissues when they live in or near smelters (Anthony and Koslowski 1982; Smith and Rongstad 1982; Beyer et al. 1985; Ma et al. 1991; Beyer and Storm 1995; Levengood and Heske 2008), mine tailings (Cooke et al. 1990; Laurinolli et al. 2006) and metal-processing industries (Johnson et al. 1978; Kisseberth et al. 1984).

Another advantage of using small mammals as sentinel species is our knowledge of their genome, permitting the development of more than 100 polymorphic microsatellite markers (Mullen et al. 2006) to evaluate genetic structure parameters.

Many organisms are exposed to complex mixtures of contaminants representing a broad spectrum of compounds. Consequently, it is likely that, when compared with humans, many animal species have far higher exposure to these substances (Hebert et al. 1996), and therefore may be ideal models for surveys that attempt to quantify genotoxic, mutagenic or ecotoxicological effects.

If we are to successfully predict ecosystem health effects, a multispecies approach for selecting sentinel organisms (different types of sentinels) is needed, and is more suitable for studying pollutant effects above the population level.

7 Conclusions and Future Perspectives

The greatest challenge in genetic ecotoxicology is to demonstrate a convincing link between contaminant effects and responses at higher levels of biological organization. The studies that have assessed biomarkers of genetic diversity in animal populations as they relate to ecosystem health are limited in number, and most of the information derived from such studies has focused on aquatic ecosystems. Moreover, a clear relationship between contaminant effects and population-level responses are often lacking, as are mechanistic explanations. Thus, the results of many studies demonstrate correlation but not causation, which suggests that despite the fact that metal contamination is present, other factors are causing the differences in the mutation rates.

The goal is to ensure reproducible and reliable results, and to produce more accurate data for providing a deeper understanding of the relationship between metal exposure and alterations in the genetic diversity of impacted populations. To achieve this, we suggest that researchers include the following parameters in each study they perform whenever possible:

1) Describe the chemical nature of each chemical pollutant in detail. In addition, the metal concentrations that appear in soil, air or water must be assessed. It is also essential to identify each of the metals involved in an exposure, rather than only referring to "metal mixtures" or "sites with heavy metal pollution".

2) Supplement any ecotoxicology data on populations with data at the community and ecosystem level. Such data are important because indicators at different levels of biological organization provide different types of information necessary for a more robust ecological risk assessment (Clements 2000). The use of biomarkers for ecotoxicological studies has become a matter of priority and should be strengthened. It is particularly important in future studies to employ biomarkers for better assessing internal doses (metal concentration in tissues, organs or biological fluids), early effects (genotoxicity assays) and susceptibility (genetic polymorphisms) in both somatic and germ cells. Integrating biomarkers into genetic ecotoxicology surveys will provide solid evidence of the ecological effects of pollution, because they may reflect metal bioaccumulation levels that exist in the population. Because of their prognostic properties, biomarkers are also useful for linking alterations at molecular and cellular levels with ecologically-relevant responses.

3) Expand the use of sentinel organisms different species in future studies. Many of the major principles underlying molecular or population genetic processes are conserved across all five kingdoms of living organisms. Therefore, it is feasible to extrapolate ecological effects that occur in a selected few species of model organisms (Theodorakis 2001), especially sentinel organisms, to all organisms. Moreover, using several sentinel organisms, a "multispecies approach", would enhance the ability to extrapolate results to higher levels of biological organization.

4) Under field conditions, the experimental design should include gradients of environmental metal contamination with several reference sites, in order to enhance the ability to identify cause-effect relationships.

5) Increase sampling of reference populations that are in close proximity to exposed populations. Such research will increase the possibility that the observed changes in the genetic structure and diversity of the exposed population are the result of exposure to a polluted environment. Sampling in close geographical proximity reduces the possibility that the observed changes between or among populations will result from phylogeographic processes.

6) Expand the use of genetic structure parameters to infer the fate of exposed populations. Bickham et al. (2000) suggested that, "because population genetic changes are expected to be independent of the mechanisms of toxicity, and sensitive indicators of transgenerational effects, they represent the ultimate biomarker of effect". Because genetic changes, especially the loss of genetic variability, might be permanent (depending on the population size and mutation rates) once variability is lost, the population cannot recover to what it was before the environmental impact. Furthermore, strong evidence suggests that genetic population diversity may be a useful biomarker of ecosystem health. For these reasons, those engaged in this emerging field of study should concentrate on finding new biomarkers, namely "biomarkers of permanent effect". Genetic variability may be used as a "biomarker of permanent effect," which we define as "measures of changes or alterations in biological or/and chemical processes that once altered will not recover or will not be the same as they were originally" (as in the case of loss of genetic variability), and will result in permanent effects on populations.

7) Seek opportunities to move ecotoxicology and biomarker research toward a more holistic approach (Chapman 2002). One such opportunity is to utilize the power of genomics as a tool to improve the understanding of toxicant impact on natural populations. In this context, "ecotoxicogenomics" will benefit from the application of high-throughput technology, in which changes in the expression of hundreds to thousands of genes (genomics), proteins (proteomics) and metabolites (metabolomics) are assessed simultaneously. Such methodologies add value to classical whole-organism testing methods, because they provide information on the molecular basis of exposure, and act as "early warning" signs that permit both more accurate classification of chemical exposures, and better prediction of the mode of action and the development of novel biomarkers. These approaches are addressed in a number of recent publications (Poynton et al. 2007; Watanabe et al. 2008; Roh et al. 2009; Villenueve et al. 2012). Moreover, these methods provide a better understanding of how to extrapolate data from the laboratory to the field and from a few sentinel species to the whole-ecosystem (Lee et al. 2008).

Finally, because genetic variability is the basis for adaptation by natural selection and is one of the pillars of biodiversity and evolution (Anderson et al. 1994; Van Straalen and Timmermans 2002), attention must be paid to any situation, such as exposure to xenobiotics.

Because demonstrating genetic, biochemical or physiological responses to toxicants may not be sufficient to protect wildlife from diversity loss or extinction, a real effort must be undertaken to discover their effects on populations, communities and ecosystems. Therefore, interdisciplinary research, along with more detailed study designs is required to solve complex environmental problems in genetic ecotoxicology.

8 Summary

Studies, in which the genetic diversity of wild populations affected by pollution are measured, provide a basis for establishing estimates of human and wildlife risks to environmental contamination and, consequently, provide a better understanding of the underlying mechanisms and the long-term effects of chemical pollution.

In this review, we summarize key aspects of the field of genetic ecotoxicology that encompasses using genetic patterns to examine metal pollutants as environmental stressors of natural animal populations. We also address the differences that exist between genetic changes from genotoxic mechanisms of action and genetic alterations that result from ecological processes, and the relationship between metal exposure and changes in the genetic diversity of chronically-exposed populations, and how the affected populations respond to environmental stress. Further, we assess the genetic diversity of animal populations that were exposed to metals, focusing on the literature that has been published since the year 2000.

Our review disclosed that the most common metals found in aquatic and terrestrial ecosystems were Cd, Zn, Cu and Pb; however, differences in the occurrence between aquatic (Cd=Zn>Cu>Pb>Hg) and terrestrial (Cu>Cd>Pb>Zn>Ni) environments were observed. Several molecular markers were used to assess genetic diversity in impacted populations, the order of the most common ones of which were SSR's >allozyme >RAPD's >mtDNA sequencing >other molecular markers.

Genetic diversity was reduced for nearly all animal populations that were exposed to a single metal, or a mixture of metals in aquatic ecosystems (except in *Hyalella azteca*, *Littorina littorea*,

Salmo trutta and *Gobio gobio*); however, the pattern was less clear when terrestrial ecosystems were analyzed.

We also address the importance and suitability of a multispecies approach to sentinel organisms in ecotoxicological studies in this review.

Finally, we proposed that future research in the topic area of this paper emphasize seven key areas of activity that pertain to the methodological design of genetic ecotoxicological studies. These points are designed to provide more accurate data and a deeper understanding of the relationship between alterations in genetic diversity of impacted populations and metal exposures. In particular that the exact nature tested chemical pollutants be clearly described, biomarkers be included, sentinel organisms be used, testing be performed at multiple experimental sites, reference populations be sampled in close geographical proximity to where pollution occurs, and genetic structure parameters and high-throughput technology be more actively employed. Furthermore, we propose a new class of biomarkers, termed "biomarkers of permanent effect", which may include measures of genetic variability in impacted populations.

Acknowledgments

P.M.G is a recipient of a fellowship from CONACYT. The authors thank Dr. Daniel Piñero Dalmau for his comments on this work.

References

Agarwal S, Sohal R (1994) DNA oxidative damage and life expectancy in houseflies. Proc Nat Acad Sci USA 91:1232–1235.

Anderson S, Sadinski W, Shugart L, Brussard P, Depledge M, Ford T, Hose J, Stegeman J, Suk W, Wirgin I, Wogan G (1994) Genetic and molecular ecotoxicology: A research framework. Environ Health Perspect 102:3–8.

Anthony R, Koslowski R (1982) Heavy metals in tissues of small mammals inhabiting waste-water-irrigated habitats. J Environ Qual 11:20–22.

Antón A, Serran T, Angulo E, Ferrero G, Rallo A (2000) The use of two species of crayfish as environmental quality sentinels: The relationship between heavy metal content, cell and tissue biomarkers and physico-chemical characteristics of the environment. Sci Total Environ 249:239-251.

Arif I, Khan H (2009) Molecular markers for biodiversity analysis of wildlife animals: A brief review. Animal Biodiv Conser 32:9-17.

Athrey N, Leberg P, Klerks P (2007) Laboratory culturing and selection for increased resistance to cadmium reduce genetic variation in the least killfish, *Heterandria Formosa*. Environ Toxicol Chem 26:1916-1921.

Baker RJ, Bickham AM, Bondarkov M, Gaschak SP, Matson CW, Rodgers BE, Wickliffe JK, Chesser RK (2001) Consequences of polluted environments on population structure: The bank vole (*Clethrionomys glareolus*) at Chornobyl. Ecotoxicology 10:211-216.

Baranski B (1987) Effect of cadmium on prenatal development and on tissue cadmium, copper, and zinc concentrations in rats. Environ Res 42:54–62.

Barret S, Khon J (1991) Genetic and evolutionary consequences of small populations in plant: Implications for conservation. In: Folk D, Holsinger K. (eds) Genetics and conservation of rare plants. Oxford University Press, Inc, New York, pp 3–30.

Battaglia B, Bisol P, Rodino E (1980) Experimental studies on the genetic effects of the marine pollution. Helgol Meer 33:587–595.

Belfiore N, Anderson S (1998) Genetic patterns as a tool for monitoring and assesment of environmental impacts: The example of genetic ecotoxicology. Environ Monit Assess 51:465–479.

Belfiore N, Anderson S (2001) Effects of contaminants on genetic patterns in aquatic organisms: A review. Mutat Res 489:97–122.

Benedetti M, Martuccio G, Fattorini D, Canapa A, Barucca M, Nigro M, Regoli F (2007) Oxidative and modulatory effects of trace metals on metabolism of polycyclic aromatic hydrocarbons in the Antarctic fish *Trematomus bernacchii*. Aquatic Toxicol 85:167–175.

Benton M, Malott M, Trybula J, Dean D, Guttman S (2002) Genetic effects of mercury contamination on aquatic snail populations: Allozyme genotypes and DNA strand breakage. Environ Toxicol Chem 21:584–589.

Berckmoes V, Scheirs J, Jordaens K, Blust R, Backeljau T, Verhagen R (2005) Effects of environmental pollution on microsatellite DNA diversity in wood mouse (*Apodemus sylvaticus*) populations. Environ Toxicol Chem 24:2898–2907.

Bernard A (2008) Biomarkers of metal toxicity in population studies: Research potential and interpretation issues. J Toxicol Environ Health Part A 71:1259–1265.

Bervoest L, Blust R (2003) Metal concentrations in water, sediment and gudgeon (*Gobio gobio*) from a pollution gradient: Relationship with fish condition factor. Env Pollut 126:9-19.

Beyer W, Pattee O, Sileo L, Hoffman D, Mulhern B (1985) Metal contamination in wildlife living near two zinc smelters. Environ Pollut Series A 38:63–86.

Beyer W, Storm G (1995) Ecotoxicological damage from zinc smelting at Palmerton, Pennsylvania. In: Hoffman DJ, Rattner BA, Burton GA, Cairns JC (eds) Handbook of ecotoxicology, CRC Press, Inc, Boca Raton, pp 596–608.

Bickham J, Smolen M (1994) Somatic and heritable effects of environmental genotoxins and the emergence of evolutionary toxicology. Environ Health Perspect 102:25–28.

Bickham J, Sandhu S, Hebert P, Chikhi L, Athwal R (2000) Effects of chemical contaminants on genetic diversity in natural populations: Implications for biomonitoring and ecotoxicology. Mutat Res 463:33–51.

Bisser J, Vogel L, Berger J, Hjelle B, Loew S (2004) Effects of heavy metals on immunocompetence of white-footed mice (*Peromyscus leucopus*). J Wildlife Dis 40:173–184.

Bourret V, Couture P, Campbell P, Bernatchez L (2008) Evolutionary ecotoxicology of wild yellow perch (*Percha flavescens*) populations chronically exposed to a polymetallic gradient. Aquat Toxicol 86:76–90.

Brown A, Hosken D, Balloux F, Bickley L, LePage G, Owen S, Hetheridge M, Tyler C (2009) Genetic variation, inbreeding and chemical exposure-combined effects in wildlife and critical considerations for ecotoxicology. Philos Trans R Soc Lond B Biol Sci 364:3377-90.

Burger J, Gochfeld M (2004) Marine birds as sentinels of environmental pollution. Ecohealth 1:263–274.

Chagnon N, Guttman S (1989) Differential survivorship of allozyme genotypes in mosquitofish populations exposed to copper or cadmium. Environ Toxicol Chem 8:319–326.

Chapman PM (2002) Integrating toxicology and ecology: putting the eco into cotoxicology. Mar Poll Bull 44:7–15.

Chen JZ, Hebert PDN (1999) Terminal branch haplotype analysis: A novel approach to investigate newly arisen variants of mitochondrial DNA in natural populations. Mutat Res-DNA Repair 434:219–231.

Clements W (2000) Integrating effects of contaminants across levels of biological organization: An overview. J Aquat Ecosyst Stress Recovery 7:113–116.

Cooke J, Andrews S, Johnson M (1990) Lead, zinc, cadmium, and fluoride in small mammals from contaminated grassland established on fluorspar tailings. Water Air Soil Pollut 51:43–54.

Coutellec M, Barata C (2011) An introduction to evolutionary processes in ecotoxicology. Ecotoxicology 20:513-23.

Depledge M (1994) Genotypic toxicity: Implications for individuals and populations. Environ Health Perspect 102:101–104.

De Wolf H, Blust R, Backeljau T (2004) The population genetic structure of *Littorina littorea* (Mollusca: Gastropoda) along a pollution gradient in the Scheldt estuary (The Netherlands) using RAPD analysis. Sci Total Environ 325:59–69.

Diamond S, Newman M, Mulvey M, Dixon P, Martinson D (1989) Allozyme genotype and time to death of mosquitofish, *Gambusia affinis* (Baird and Girard), during acute exposure to inorganic mercury. Environ Toxicol Chem 8:613–622.

Diamond S, Newman M, Mulvey M, Guttman S (1991) Allozyme genotype and time-to-death of mosquitofish, *Gambusia holbrooki*, during acute inorganic mercury exposure: A comparison of populations. Aquat Toxicol 21:119–134.

Dimsoski P, Toth G (2001) Development of DNA-based microsatellite marker technology for studies of genetic diversity in stressor impacted populations. Ecotoxicology 10:229–232.

D´Surney S, Shugart L, Theodorakis C (2001) Genetic markers and genotyping methodologies: An overview. Ecotoxicology 10:201–204.

Duan Y, Guttman S, Oris J, Bailer A (2001) Differential survivorship among allozyme genotypes of *Hyalella azteca* exposed to cadmium, zinc or low pH. Aquat Toxicol 54:15–28.

Durrant ChJ, Stevens JR, Hogstrand C, Bury NR (2011) The effect of metal pollution on the population genetic structure of brown trout (*Salmo trutta* L.) residing in the River Hayle, Cornwall, UK. Environ Pollut 159:3595–3603.

Eeva T, Belskii E, Kuranov B (2006) Environmental pollution affects genetic diversity in wild bird populations. Mutat Res 608:8–15.

Ehrenfeld D (1992) Ecosystem health and ecological theories. In: Norton BG, Heskell B (eds) Ecosystem health. New goals for environmental management, Island Press, Washington DC, pp135–143.

Eisler R (1997) Zinc hazards to plants and animals with emphasis on fishery and wildlife resources. In: Chemisinoff PN (ed) Ecological issues and environmental impact assessment, Gulf Publ Co, Houston, pp. 443–537.

Ekschmitt K, Korthals G (2006) Nematodes as sentinels of heavy metals and organic toxicants in the soil. J Nematol 38:13–19.

Endler J (1995) Multiple-trait coevolution and environmental gradients in guppies. Trends Ecol Evol 10:22–29.

EPA Environmental Protection Agency (2000) Innovative remediation technologies: Field scale demonstration projects in North America.

Evenden AJ, Depledge MH (1997) Genetic susceptibility in ecosystems: The challenge for ecotoxicology. Environ Health Perspect 105:849–854.

Fratini S, Zane L, Ragionieri L, Vannini M, Cannicc S (2008) Relationship between heavy metal accumulation and genetic variability decrease in the intertidial crab *Pachygrapsus marmoratus* (Decapoda; Grapsidae). Est Coast Shelf Sci 79:679–686.

Gardeström J, Dahl U, Kotsalainen O, Maxson A, Elfwing T, Grahn M, Bengtsson B, Breitholtz M (2008) Evidence of population genetic effects of long-term exposure to contaminated sediments: A multi-endpoint study with copepods. Aquat Toxicol 86:426–436.

Gillespie R, Guttman SI (1989) Effects of contaminants on the frequencies of allozymes in populations of the central stoneroller. Environ Toxicol Chem 8:309–317.

Guttman S (1994) Population genetic structure and ecotoxicology. Environ Health Perspect 102:97–100.

Haimi J, Knott K, Selonen S, Laurikainen M (2006) Has long-term metal exposure induced changes in life history traits and genetic diversity of the enchtraeid worm *Cognettia sphagnetorum*. Environ Poll 140:463–470.

Harper-Arabie R, Wirth E, Fulton M, Scott G, Ross P (2004) Protective effects of allozyme genotype during chemical exposure in the grass shrimp, *Palaemonetes pugio*. Aquat Toxicol 70:41–54

Hebert PM, Murdoch-Luiker M (1996) Genetic effects of contaminant exposure-towards an assessment of impacts on animal populations. Sci Total Environ 191:23–58.

Hoffman A, Daborn P (2007) Towards genetic markers in animal populations as biomonitors for human-induced environmental change. Ecol Lett 10:63–76.

Hoffmann A, Willi Y (2008) Detecting genetic responses to environmental change. Nat Rev Genet 9:421–429.

Husby M, Hausbeck J, McBee K (1999) Chromosomal aberrancy in white-footed mice (*Peromyscus leucopus*) collectd on abandoned coal strip mines, Oklahoma, USA. Environ Toxicol Chem 18:919–925.

Johnson M, Roberts R, Hutton M (1978) Distribution of lead, zinc, and cadmium in small mammals from polluted environments. Oikos 30:153–9.

Jordaens K, De Wolf H, Van Houtte N, Vandecasteele B, Backeljau T (2006) Genetic variation in two land snails Cepaea nemoralis and Succinea putris (Gastropoda, Pulmonata), from sites differing in heavy metal content. Genetica 128:227-239.

Keane B, Collier M, Rogstad S (2005) Pollution and genetic structure of North American populations of the common dandelion (*Taraxacum officinale*). Environ Monit Assess 105:341–357.

Kim S, Rodriguez M, Suh J, Song J (2003) Emergent effects of heavy metal pollution at a population level: *Littorina brevicula* a study case. Mar Pollut Bull 46:74–80.

Kisseberth W, Sunderberg J, Nyboer R, Reynolds J, Kasten S, Beasley V (1984) Industrial lead contamination of an Illinois wildlife refuge and indigenous small mammals. J Am Vet Med Assoc 185:1309–1313.

Laurinolli M, Bendell-Young L (2006) Copper, zinc, and cadmium concentrations in *Peromyscus maniculatus* sampled near an abandoned copper mine. Environ Contam Toxicol 30:481–486.

Lee C, Newman M, Mulvey M (1992) Time to death of mosquitofish (*Gambusia holbrooki*) during acute inorganic mercury exposure: Population structure effects. Arc Environ Contam Toxicol 22:284–287.

Lee J.S, Raisuddin S, Schlenk D. (2008) Kryptolebias marmoratus (Poey, 1880): a potential model species for molecular carcinogenesis and ecotoxicogenomics. Journal of Fish Biology 72, 1871–1889.

Levengood J, Heske E (2008) Heavy metal exposure, reproductive activity, and demographic patterns in white-footed mice (*Peromyscus leucopus*) inhabiting a contaminated wetland. Sci Total Environ 389:320–328.

Loveless M, Hamrick J (1984) Ecological determinants of genetic structure in plant population. Annu Rev Ecol Syst 15:65–95.

Lowe A, Harris S, Ashton P (2004) Ecological genetics: Design, analysis and application. Blackwell Publishing, Oxford, 313 p.

Lucchini V (2003) AFLP: A useful tool for biodiversity conservation and management. Comptes Rendus Biol 326:43-48.

Lynch M, Conery U, Bürger R (1995) Mutation accumulation and the extinction of small population. Am Nat 146:489–518.

Ma W, Denneman W, Faber J (1991) Hazardous exposure of ground-living small mammals to cadmium and lead in contaminated terrestrial ecosystems. Arch Environ Contam Toxicol 20:266–270.

Ma X, Cowles D, Carter R (2000) Effect of pollution on genetic diversity in the Bay Mussel *Mytilus galloprovincialis* and the acorn bernacle *Balanus gladula*. Mar Environ Res 50:559–563.

Maes G, Raeymaekers J, Pampoulie C, Seynaeve A, Goemans G, Belpaire C, Volckaert F (2005) The catadromous European eel *Anguilla anguilla* (L.) as a model for freshwater evolutionary ecotoxicology: Relationship between heavy metal bioaccumulation, condition and genetic variability. Aquat Toxicol 73:99–114.

Markert B, Wappelhorst O, Weckert V, Herpin U, Siewers U, Friese K, Breulmann G (1999) The use of bioindicators for monitoring the heavy-metal status of the environment. J Radioanalytical Nuclear Chem 240:425–429.

Matson C, Lambert M, McDonald T, Autenrieth R, Donnelly K, Islamzadeh A, Politov D, Bickham J (2006) Evolutionary toxicology: Population-level effects of chronic contaminant exposure on the marsh frogs (*Rana ridibunda*) of Azerbaijan. Environ Health Perspect 114:547–552.

Medina M, Correa J, Barata C (2007) Micro-evolution due to pollution: Possible consequences for ecosystem responses to toxic stress. Chemosphere 67:2105–2114.

Moore M, Depledge M, Readman J, Leonard D (2004) An integrated biomarker-based strategy for ecotoxicological evaluation of risk in environmental management. Mutat Res 552:247–268.

Morgan AJ, Killie P, Sturzenbaum SR (2007) Microevolution and ecotoxicology of metals in invertebrates. Environ Sci Technol 41:1085–1096.

Mullen L, Hirschmann R, Prince K, Glenn T, Dewey M, Hoekstra H (2006) Sixty polymorphic microsatellite markers for the oldfield mouse developed in *Peromyscus polionotus* and *Peromyscus maniculatus*. Mol Ecol Notes 6:36–40.

Nedjoud G, Houria B, Rachid R, Amira D, Reda D (2009) Impact of pollution by industrial metallic dust on bio-accumulator organism *Helix aspersa*. Global Veterinaria 3:276–80.

Nevo E, Shimony T, Libni M (1978) Pollution selection of allozyme polymorphisms in Barnacles. Experientia 34:1562–1564.

Nevo E, Lavie B, Ben-Shlomo R (1983) Selection of allelic isozyme polymorphisms in marine organisms: Patern, theory and application. In: Ratazzi MC, Scandalios JC, Whitt GS (eds)

Isozymes: Current topics in biological and medical research. Genetics and Evolution AR Liss, New York, pp 69–92.

Newman M, Diamond S, Mulvey M, Dixon P (1989) Allozyme genotype and time to death of mosquitofish, *Gambusia affinis* (Baird and Girard) during acute toxicant exposure: A comparison of arsenate and inorganic mercury. Aquat Toxicol 15:141–156.

Pascoe G, Blanchet R, Liner G (1994) Bioavailability of metals and arsenic to small mammals at a mining waste-contaminated wetland. Arch Environ Contam Toxicol 27:44–50.

Patarnello T, Guinez R, Battaglia B (1991) Effects of pollution on heterozygosity in the bernacle *Balanus amphitrite* (Cirripedia: Thoracica). Mar Ecol Prog Ser 70:237–243.

Peles JD, Towler WI, Guttman SI (2003) Population genetic structure of earthworms (*Lumbricus rubelluz*) in soils contaminated by heavy metals. Ecotoxicology 12:379–386.

Poynton H, Varshavky J, Chang B, Cavigiolio G, Chan S, Holman P, Loguinov A, Bauer D, Komachi K,Theil E, Perkins E, Hughes O, Vulpe C (2007) Daphnia magna Ecotoxicogenomics provides mechanistic insights into metal toxicity. Environ Sci Technol 47:1044–1050.

Roark S, Andrews J, Guttman S (2001) Population genetic structure of the western mosquitofish *Gambusia Affinis,* in a highly channelized portion of the San Antonio River in San Antonio, TX. Ecotoxicology 10:223–227.

Rogstad S, Keane B, Collier M (2003) Minisatellite DNA mutation rate in dandelions increases with leaf-tissue concentrations of Cr, Fe, Mn, and Ni. Environ Toxicol Chem 22:2093–2099.

Roh J, Sim S.J, Yi J, Park K, Chun K, Ryu D, Choi J (2009) Ecotoxicity of silver nanoparticles on the soil nematode Caenorhabditis elegans using functional ecotoxicogenomics. Env Sci Technol 43:3933–3940.

Ross K, Cooper N, Bidwell J, Elder J (2002) Genetic diversity and metal tolerance of two marine species: A comparison between populations from contaminated and reference sites. Mar Poll Bull 44:671–679.

Scheirs J, De Coen A, Covaci A, Beernaert J, Kayawe V, Caturla M, De Wolf H, Baert P, Van Oostveldt P, Verhagen R, Blust R, De Coen W (2006) Genotoxicity in wood mice (*Apodemus sylvaticus*) along a pollution gradient: Exposure age and gender related effects. Environ Toxicol Chem 25:2154–2162.

Smith G, Rongstad O (1982) Small mammal heavy metal concentrations from mined and control sites. Environ Poll 28:121–134.

Staton J, Schizas N, Chandler G, Coull B, Quattro J (2001) Ecotoxicology and population genetics: The emergence of "phylogeographic and evolutionary ecotoxicology". Ecotoxicology 10:217–222.

Storelli M, Marcotrigiano G (2005) Bioindicator organisms: Heavy metal pollution evaluation in the ionian sea (Mediterranean Sea-Italy). Environ Monit Assess 102:159–166.

Sues B, Tarashewski H, Rydlo M (1997) Intestinal fish parasites as heavy metal bioindicators: A comparison between *Acanthocephalus lucci* and zebra mussel, *Dressena polymorpha*. Bull Environ Contam Toxicol 59:14–21.

Sunderberg J, Okarsson A (1992) Placental and lactational transfer of mercury from rats exposed to methylmercury in their diet: Speciation of mercury in the offspring. J Trace Elem Exp Med 5:47–56.

Talmage S, Walton B (1991) Small mammals as monitors of environmental contaminants. Rev Environ Contam Toxicol 119:47–145.

Theodorakis C, Swartz C, Rogers W, Bickham J, Donnely K, Adams S (2000) Relationship between genotoxicity, mutagenicity, and fish community structure in a contaminated stream. J Aquat Ecosyst Stress Recovery 7:131–43.

Theodorakis C (2001) Integration of genotoxic and population genetic endpoints in biomonitoring and risk assessment. Ecotoxicol 10:245–256.

Theodorakis CW, Bickham JW, Lamb T, Medica PA, Barret LT (2001) Integration of genotoxicity and population genetic analyses in kangaroo rats (*Dipodomys merriami*) exposed to radionuclide contamination at the nevada test site. USA. Environ Toxicol Chem 20:317–326.

Tremblay A, Lesbarreres D, Merritt T, Wilson C (2008) Genetic structure and phenotypic plasticity of yellow perch (*Perca flavescens*) populations influences by habitat, predation, and contamination gradients. Integr Environ Assess Manag 4:264–266.

Ungherese G, Mengoni A, Somigli S, Baroni D, Focardi S, Ugolini A (2010) Relationship between heavy metals pollution and genetic diversity in Mediterranean population of the sandhopper *Talitrus saltator* (Montagu) (Crustaceae, Amphipoda). Environ Pollut 158:1638–1643.

Van Straalen N (1999) Genetic biodiversity in toxicant-stressed populations. Prog Environ Sci 1:195–201.

Van Straalen N, Timmermans M (2002) Genetic variation in toxicant-stressed populations: An evaluation of the "genetic erosion" hypothesis. Hum Ecol Risk Assess 8:983–1002.

Villenueve D, Garcia-Reyero N, Escalon B, Jensen K, Cavallin J, Makynen E, Durhan E, Kahl M, Thomas L, Perkins E, Ankley G (2012) Ecotoxicogenomics to Support Ecological Risk Assessment: A Case Study with Bisphenol A in Fish. Env Sci Technol 46:51–59.

Watanabe H, Kobayashi K, Kato Y, Oda S, Abe R, Tatarazako N, Iguchi T (2008) Transcriptome profiling in crustaceans as a tool for ecotoxicogenomics. Cell Biol Toxicol 24: 641–647.

Weinstein I (1988) The origins of human cancer: Molecular mechanism of carcinogenesis and their implications for cancer prevention and treatment. Cancer Res 48: 4135–4143.

WHO. World Health Organization (2007) Health risks of heavy metals from long range transboundary air pollution, European committee.

Yauk C, Quinn J (1996) Multilocus DNA fingerprinting reveals high rate of heritable genetic mutation in herring gulls nesting in an industrialized urban site. Proc Natl Acad Sci USA 93:12137–12141.

Yauk C, Fox G, McCarry B, Quinn J (2000) Induced minisatellite germline mutations in herring gulls (*Larus argentatus*) living near steel mills. Mutat Res 452:211–218.

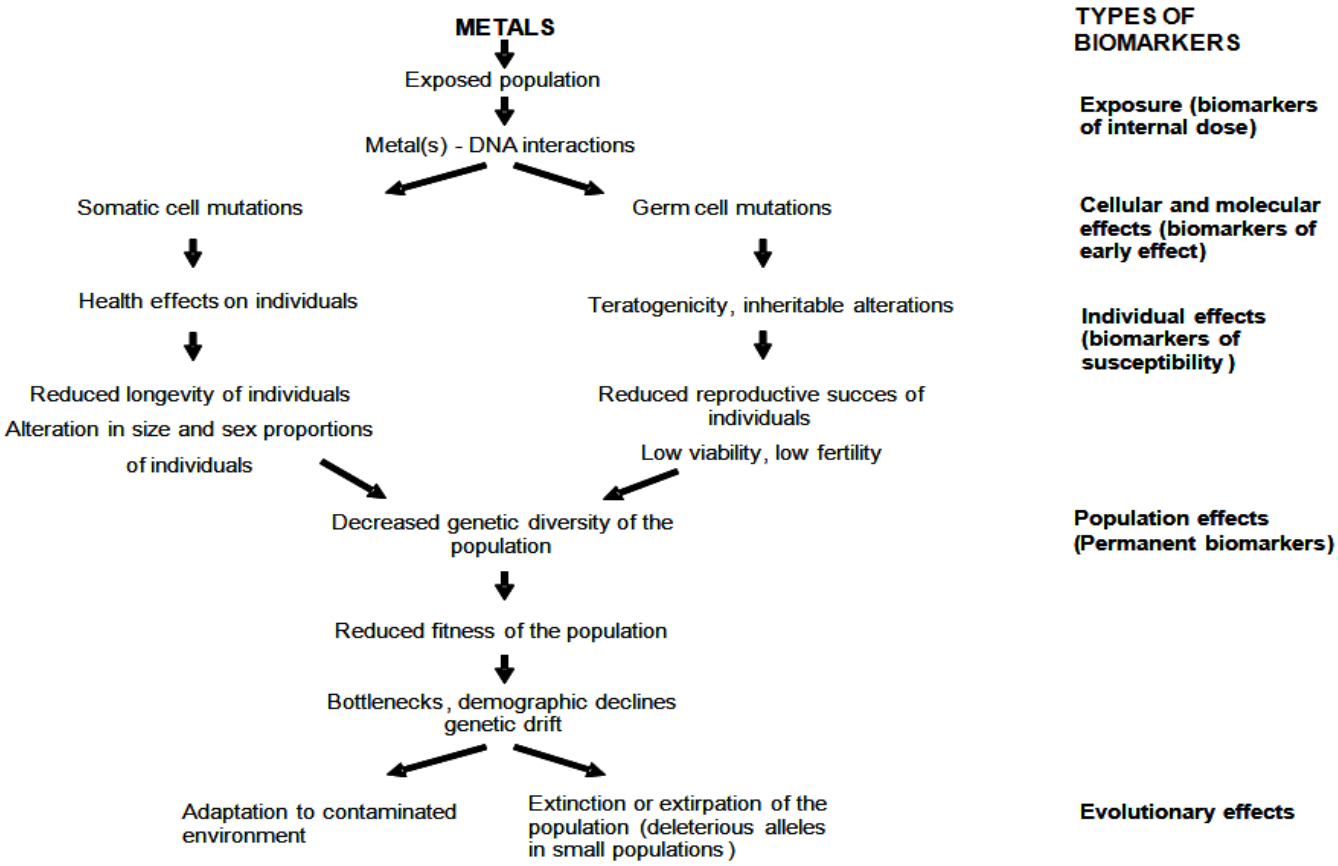

Fig. 1. Schematic representation of the relationships between the processes leading to decreased genetic diversity in animal populations exposed to metal pollution. Modified from Guttman (1994), Theodorakis (2001) and Staton et al. (2001).

Table 1 Studies published since the year 2000 are summarized, in which the genetic diversity in animal populations exposed to metal pollution are addressed.

SPECIES (common and scientific name)	CLASSIFICATION (Phylum: Order)	GENETIC DIVERSITY AND STRUCTURE PARAMETERS	CHANGES IN GENETIC DIVERSITY PATTERNS	TECHNIQUE	STUDY CONDITIONS	TYPE OF METAL AND CONCENTRATION	COMMENTS	REFERENCE
	AQUATIC ECOSYSTEM							
Acorn bernacle (*Balanus glandula*)	Arthropoda: Sessilia	Diversity indexes, diversity by rarefaction analysis, proportion of shared haplotypes	Genetic diversity **decreased** significantly in exposed populations	RAPD	Field: levels of pollutants in the sediment (N.R.)	Heavy metal mixture (N.R.)	Pollution at the impacted sites may reduce genetic diversity among aquatic invertebrate populations. Individuals at impacted sites were more likely to share haplotypes than were those from reference sites	Ma et al. 2000
Amphipod (*Hyalella azteca*)	Arthropoda: Amphipoda	Genetic distance, genetic variation at different loci	**No relationship** was found between levels of heterozygosity and metal exposure (was not formally evaluated using statistical methods). Increased genetic divergence.	Allozyme	Laboratory: One-month-old *H. azteca* were used to evaluat each metal: Cd (µg/L) and Zn (mg/L)	Water: (mean±S.D.) Cd (37.10±2.52), Zn (5.77±0.30)	Suggests the potential use of genetic distance as a water bioindicator	Duan et al. 2001
Prawn (*Leander intermedius*)	Arthropoda: Decapoda	BSI	**Decreased** genetic diversity of prawn population in polluted site vs. one reference population, and not significantly lower than that of all reference populations.	RAPD	Laboratory: levels of trace metals in food pellets in one contaminated site (µg/g). Prawns were fed metal mixtures in varing concentrations ranging from 0.14 to 26 times that found in contaminated site	Food pellets: (range) Cd (7.8-4580), Zn (0-136197), Cu (18.9-1975), Pb (0-34552), Mn (0-22359)	These observations highlight the need to include various reference populations in genetic population studies and the need to assess the influence of pollution on the genetic diversity of more than one species	Ross et al. 2002
Isopod (*Platynympha longicaudata*)	Arthropoda: Isopoda	BSI	**Decreased** genetic diversity of isopods populations in polluted sites vs. all reference populations	RAPD	Laboratory: levels of trace metals in food pellets in one contaminated site (µg/g). Isopods were fed metal mixtures in varing concentrations ranging from 0.14 to 26 times that found in contaminated site	Food pellets: (range) Cd (7.8-4580), Zn (0-136197), Cu (18.9-1975), Pb (0-34552), Mn (0-22359)	These observations highlight the need to include various reference populations in genetic population studies and the need to assess the influence of pollution on the genetic diversity of more than one species	Ross et al. 2002
Harpacticoid copepod (*Attheyella crassa)*	Arthropoda: Harpacticoida	Fixation index, heterozygosity	**Decreased** genetic diversity in experimental groups	AFLP	Field: analyzed metals in surface sediments (top 0.5-1cm) in two polluted sites (µg/g dry wt)	Sediment: (range) Zn (1110-2966), Cu (614-1039), Pb (55-1080), Hg (n.a.-28.6)	Toxicant exposure can reduce genetic diversity and cause population differentiation	Garderstron et al. 2008
Periwinkle (*Littorina littorea*)	Mollusca: Mesogastropoda	Heterozygosity, Fst	**No relationship** was found between levels of locus-specific heterozygosity and metal exposure.	RAPD	Field: levels of pollutants in soft tissue, in seven sites (µg/g-^{1} dry wt.)	Soft tissue: (range) Ag (0.81-4.85), As (7.96-22.92), Cd (0.92-5.23), Co (0.0-2.15, Cr (0.11-1.48), Cu (68.2-176.0), Fe (337.0-1214.0), Mn (19.64-113.7), Ni (3.43-7.43), Pb (0.86-1.67), Zn (59.6-106.0)	Authors suggest that selection, rather than bottleneck effects, induced by less favourable leaving conditions at polluted sites are responsible for the genetc patterning	De Wolf et al. 2004

Gasteropod (*Littorina brevicula*)	Mollusca: Mesogastropoda	Haplotype distribution and diversity	**Decreased** genetic diversity in experimental group. Haplotype diversity was significantly lower in polluted environments	Sequencing analysis (mtDNA)	Field: analyzed metals in seawater (µl/l), sediment (µg/g) and organism (µg/g) in three polluted sites	Seawater: (range) Cd (0.010-1.705), Zn (1.70-35.12), Cu (0.61-24.6), Pb (0.042-0.120). Sediment: (range) Cd (2.71-11.2), Zn (144-230), Cu (37.6-47.8), Pb (41.0-56.8). Organism: Cd (11.81), Zn (88), Cu (126), Pb (1.76)	Observed emergent effects from pollution at a population level, taking into account rare haplotypes	Kim et al. 2003
Snail (*Pleurocera canaliculatum)*	Mollusca: Caenogastropoda	Allele and genotype frequencies, heterozygosity	**Decreased** heterozygosity in experimental group vs. reference group	Allozyme	Field: whole-body of *P. canaliculatum* from five sites (µg/g dry wt)	Whole-body: (range) Hg (0.678-4.257)	Reinforces the use of allozyme analysis as a marker of contamination and possible selection for pollution resistance.	Benton et al. 2002
Bay mussel (*Mytilus galloprovinciali*)	Mollusca: Mytiloida	Diversity indexes, diversity by rarefaction analysis, proportion of shared haplotypes	Genetic diversity **decreased** significantly in exposed populations	RAPD	Field: levels of pollutants in the sediment (N.R.)	Heavy metal mixture (N.R.)	Pollution at the impacted sites may reduce genetic diversity among aquatic invertebrate populations. Individuals at impacted sites were more likely to share haplotypes than were those from reference sites	Ma et al. 2000
Marsh frogs (*Rana ridiunda*)	Chordata: Anura	Haplotype and neuclotide diversity, Fst, Nm	Genetci diversity **decreased** significantly in exposed populations	Sequencing analysis (mtDNA)	Field: March frogs were collected from of eight exposed and three reference sites. Hg concentrations in fresh water sediments (ppm)	Fresh water sediments Hg (1.49) and a complex mixtire of chemical pollutants	The authors conclude that the observed loss of diversity is likely the results of population declines, and thet environmental degradation is most likely cause of the regional reductions of genetic diversity	Matson et al. 2006
Yellow perch (*Perca flavescens*)	Chordata: Perciformes	Heterozygosity, allelic diversity and richness, Fst, Rst and IR	Genetic diversity **decreases** along a gradient of increasing Cd and Cu contamination . IR: presented the opposite tendency as the more contaminated individuals were more diverse than were the less contaminated ones in contaminated and reference populations.	SSR's	Field: liver of *P. flavescens* from 20 sites (µg/g dry wt)	*Liver: (range) Cd (2-37), Cu (20-185)	Chronic exposure to metal contamination have impacted genetic diversity among populations of wild yellow perch, which may affect the capacity of populations to respond to environmental changes	Bourret et al. 2008
Brown trout (*Salmo trutta*)	Chordata: Salmoniformes	Allelic richness , Fst, Ho and He, Nm	Genetic diversity estimates **did not support** a negative correlation between population genetic diversity and increasing metal pollution	SSR's	Field and Laboratory: water metal concentrations ($\mu g/L^{-1}$).	Water: (two conditions) 1) Cu (94), Zn (760) 2) Cu (4), Zn (28)	Population genetic analysis indicated that metals were not a barrier to gene flow within the river. The metal tolerance trait exhibited by this Brown trout population may represent an important component of the species genetic diversity in the region	Durrant et al. 2011
Gudgeon (*Gobio gobio*)	Chordata: Cypriniformes	Na, Ho, He, genetic and genotypic differentiation	Ho, He, and Na **did not differ** between contaminated and reference sites.	Allozyme and SSR's	Field: metal concetrations of the surface water in four sites were analyzed (µg/L)	Surfece water: (range) Cd (20- 30), Zn (1500-2100)	Long-term exposure to metals can induce changes at the population genetic level in natural fish populations, which can be detected both at microsatellite as well as at allozyme loci	Bervoets and Blust 2003
Least killifish (*Heterandria formosa*)	Chordata: Cyprinodontiformes	Heterozigosity	**Decreased** genetic diversity in three experimental populations vs. reference populations, only two decreased significantly	SSR's	Laboratory: Immature fish were exposed to Cd (mg/L) until at least 50% of individuals had died	Water: Cd (6)	Loss of genetic variation in Lab populations is taken into account when extrapolating from lab to natural populations	Athrey et al. 2007

Table 1. Continued.

European eel (*Anguilla anguilla*)	Chordata: Anguilliformes	Allele and genotype frequencies, levels of polymorphisms and heterozygosity	**Decreased** genetic variability in strongly polluted eels	Allozyme and SSR's	Field: heavy metals in muscle tissue of *A. anguilla* from 16 sites in three rivers. Concentration were expressed in μg kg $^{-1}$ (Hg, Cd, Pb, Ni, Cr, As and Se) or mg kg $^{-1}$ (Cu and Zn) wet weight	Muscle tissue: (range) Cd (1.5-23.1), Zn (17.0-32.5), Cu (0.3-1.2), Pb (5.0-95.3), Hg (59.6-245.2), Ni (5.0-94.0), Cr (135.3-823.7), As (135.0-704.0), Se (329.0-1556.0)	Significant and negative correlation between metals and fitness, suggesting an impact of pollution on the health of subadult eels	Maes et al. 2005
TERRESTRIAL ECOSYSTEM								
Earthworms (*Lumbricus rubellus*)	Annelida: Haplotaxida	Allele and genotype frequencies, heterozygosity	**Increased** heterozygosity in exposed compared to reference individuals. Allele and genotypic frequencies **did not differ** between groups	Electrophoretic analysis	Field: Concentration of heavy metals in soils (ppm) were monitored yearly in each plot from 1978 to 1993	Soil: (range) Cd (1.3-2.7), Zn (81.0-140.5), Cu (16.9-36.0), Pb (23.1-48.0)	Certain alleles and genotypes may be more sensitive to the effects of heavy metals	Peles et al. 2003
Enchytraeid worm (*Cognettia sphagnetorum)*	Annelida: Haplotaxida	Allele and genotype frequencies, H, D	**Decreased** genetic diversity in experimental group (H, unique genotypes, except D)	Allozyme	Laboratory: soil used in the laboratory experiments (after 15 weeks incubation) with Cu (mg kg-1 dry matter)	Soil: (mean±S.E.) Total Cu (2000±30.1) Extractable Cu (183±3.3)	Greater diversity and more unique genotypes in the population living in the uncontaminated site	Haimi et al. 2006
Land snail (*Cepaea nemoralis*)	Mollusca: Stylommatophora	Polymorphic loci, allelic richness, Ho, He, ϕ	**Increased** observed heterozygosity (Ho) in exposed compared to reference plots	Allozyme	Field: Concentration of heavy metals in sediment soils (mg kg-^{1} dry soil)	Soil: (mean±D.E.) Cd (9.7±6.4), Cr (269±209), Cu (190±89), Ni (95±18), Pb (397±249), Zn (1520±846)	Observed patterns of genetic variation may be explained by the action of genetic drift, pollution-mediated selection, restricted gene flow, or a combination of these processes	Jordaens et al. 2006
Land snail (*Succinea putris*)	Mollusca: Stylommatophora	Polymorphic loci, allelic richness, Ho, He, ϕ	Genetic diversity **is not affected** by metal pollution.	Allozyme	Field: Concentration of heavy metals in sediment soils (mg kg-^{1} dry soil)	Soil: (mean±D.E.) Cd (9.7±6.4), Cr (269±209), Cu (190±89), Ni (95±18), Pb (397±249), Zn (1520±846)	Observed patterns of genetic variation may be explained by the action of genetic drift, pollution-mediated selection, restricted gene flow, or a combination of these processes	Jordaens et al. 2006
Amphipod sandhopper (*Talitrus saltador*)	Arthropoda: Amphipoda	Average gene diversity over loci mean number of pair- wise differences	**Decreased** in Hg exposed populations. Population from sites with High Hg availability had the lowest values of genetic diversity	flSSR´s	Field: metal concetrations in tissue of *A. sandhopper* (ppm) and sand (ppm) from eight sites were analyzed.	Sandhopper: (range) Cd (0.40-1.74), Cu (40.6-73.6), Hg (0.07-0.21) *Sand: (range) Cd (0.01-0.24), Cu (1.0-4.0), Hg (0.02-0.11)	Validate the use of fl-SSR markers in genetic studies in sandhoppers and support the "genetic erosion hypothesis" by showing the negative influences of Hg contamination on genetic diversity	Ungherese et al. 2010
Crab (*Pachygrapsus marmoratus*)	Arthropoda: Decapoda	Ho, He, allelic richness, number of private alleles, standardized mean d^{2} (parental similarity)	**Decreased** genetic variability in *P. marmoratus* from polluted sites. A significanly lower percentage of unrelated individuals, than populations from unpolluted sites.	SSR's	Field: metal concentration in gills and hepatopancreas in *P. marmoratus* adult males (μg/g wet wt)	Gills: (approx) As (5), Pb (2.2), Cd (0.2), Cu (75) Hepatopancreas: (approx) As (20), Pb (1.4), Cd (0.48), Cu (340)	This study supports the "genetic erosion" hypothesis for metal heavy exposure in natural environments	Fratini et al. 2008
Insectivourus paserines (*Ficedula hypoleuca*)	Chordata: Passeriformes	Nucleotide diversity	**Decreased** nucleotide diversity in *F. hypoleuca* in polluted sites	Sequencing analysis (mtDNA)	Field: metal concentration in feathers of *F. hypoleuca* from three sites were analyzed (μg/g dry wt)	Fearther: (range) Cd (0.04-0.17), Zn (134.4-185.4), Cu (11.6-15.3), Pb (1.02-23.6), Ni (3.82-12.9), Al (39.1-53.74), As (0.24-8.41), Cr (0.90-2.96), Sn (0.72-1.28)	Genetic diversity depends on species and their metabolism	Eeva et al. 2006

Table 1. Continued.

Pied flycatcher (*Parus major*)	Chordata: Passeriformes	Nucleotide diversity	**Increased** nucleotide diversity in *P. major* in polluted sites suggesting high mutation rates	Sequencing analysis (mtDNA)	Field: heavy metal concentration in feathers of *P. major* from one site were analized.	Feather: (mean ± S.E.) Cd (0.03±0.00), Zn (132.4±2.56), Cu (15.9±1.15), Pb (2.00±0.64), Ni (9.18±1.06), Al (45.5±3.92), As (1.10±1.10), Cr (0.82±0.06), Sn (3.44±0.20)	Genetic diversity depends on species and their metabolism	Eeva et al. 2006
Herring gulls (*Larus argentatus*)	Chordata: Charadriiformes	Mutation rates, number of bands scored	Mutation rates **increase** significantly in steel sites vs. urban and rural sites	Multilocus DNA fingerprinting	N.R.	Steel mills	Demonstrate significant risk for induced germ line mutations in zones with steel operations	Yauk et al. 2000
Wood mouse (*Apodemos silvaticus*)	Chordata: Rodentia	Heterozygosity, allele richness, Fst, gene flow	Genetic diversity **is not affected** by metal pollution. Gene flow among populations restricted	SSR's	Field: heavy metals in soil from seven sites. Concentration were expressed in µg/g (Ag, As, Cd, Co, Cr, Cu, Mn, Ni, Pb, and Zn) or mg/g (Al and Fe) dry weight	Soil: (range) Cd (0.07-26.25), Zn (4.19-224.66), Cu (2.64-133.89), Pb (18.18-693.27), Ni (0.93-15.87), Al (0.69-13.70), Ag (0.06-4.02), As (3.25-26.25), Co (0.32-6.71), Cr (2.36-113.78), Mn (4.10-107.87), Fe (1.17-59.39)	Genetic diversity in the wood mouse populations is not affected by the heavy metal pollution. Pollution induced stress is not intense enough, or insufficient time has passed since the onset of pollution stress to induce a population genetic response.	Berckmoes et al. 2005

RAPD = random amplified polymorphic DNA, AFLP = amplified fragment length polymorphism, SSR's= sequence simple repeats or microsatellite markers, BSI = band-sharing index, IR = internal relatedness, fISSR's = fluorescence inter-simple sequence repeat, N.R. = not reported, Fst, Rst and ϕ = genetic differentiation, Ho = observed heterozygocity, He = expected heterozygocity, Na = number of alleles per locus, H = Shannon-Wiener index, D = Simpson index, Nm = number of migrants, * = approximate data (data represented by bar column plots in the original reference).