UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE CC. BIOLÓGICAS
Departamento de Ecología

INCIDENCIA DE LOS CONTAMINATES AMBIENTALES GENOTÓXICOS EN CÉLULAS DE TRUCHA ARCOIRIS (ONCORHYNCHUS MYKISS)

MEMORIA PRESENTADA PARA OPTAR AL GRADO DE DOCTOR POR Concepción Becerril Moral

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Concepción Becerril Moral.

Marzo, 2002

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Tesis dirigida por la Dra. Argelia Castaño Calvo y presentada por Concepción Becerril Moral para optar al grado de Doctor en Ciencias Biológicas por la Universidad Complutense de Madrid.

Vº Bº

Fdo.: Argelia Castaño Calvo

Fdo.: Concepción Becerril Moral

A mis padres

A Antonio, Elena y Miguel

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1. INTRODUCCIÓN

El desarrollo económico producido en el ultimo siglo ha estado íntimamente ligado a la utilización de una gran cantidad de productos químicos en todos los campos de la actividad humana. La liberación continuada de los mismos, o sus derivados, al ambiente ha generado problemas de contaminación a escala mundial que afectan tanto a la salud humana como al mantenimiento de los ecosistemas.

El alcance global del deterioro ambiental fue puesto en evidencia ya en los años sesenta. Poco a poco se fue tomando conciencia de que los recursos del planeta son limitados, y que era necesario controlar la actividad industrial mundial y aunar esfuerzos para minimizar los efectos adversos de la contaminación. Estos dos objetivos se plantearon como retos sin precedentes asumiendo que en cualquier actividad humana no existe el riesgo cero.

Consecuencia de ello fue la creación de una estrategia mundial para la conservación de la naturaleza como pieza clave en la consecución de un *desarrollo sostenible* estableciéndose de forma explicita, por primera vez, la existencia de una interrelación dinámica entre economía y medioambiente.

La respuesta institucional a esta nueva concepción se recogió en el Convenio sobre la Diversidad Biológica firmado en la Conferencia de las Naciones Unidas de Medio Ambiente y Desarrollo, celebrada en Río de Janeiro en 1992. Después de la cumbre de Río quedaron establecidos los principios que deben regir las actuaciones medioambientales en materia de contaminación, y que pueden resumirse en: el *Principio de precaución, el Principio de quien contamina paga*, y el compromiso de todos los Estados de *aplicar las mejores técnicas disponibles y las mejores prácticas medioambientales*, tomando las medidas necesarias para conseguir un ambiente saludable en el marco de un desarrollo sostenible.

La gestión y conservación de los sistemas naturales se ha incorporado a las políticas de los diferentes países de la OCDE (Organización para la Cooperación y Desarrollo Económico), estableciéndose el desarrollo conjunto de programas de investigación y la elaboración de normativas de gestión medioambiental.

1.1 CONCEPTOS GENERALES DE LA VALORACIÓN DE RIESGO MEDIOAMBIENTAL.

El concepto de riesgo medioambiental se puede definir como la probabilidad de que el uso de una sustancia química o actividad humana pueda producir efectos adversos sobre la estructura y función de los ecosistemas, entendiendo éstos como unidades discretas formadas por un conjunto de factores abióticos y bióticos que interactúan para formar un sistema estable.

(Smrchek and Zimamm 1998).

Los procedimientos de valoración de riesgo se realizan mediante la identificación y cuantificación de estos efectos adversos, y constituyen una poderosa herramienta para la toma de decisiones en el proceso de gestión medioambiental. Dichos procedimientos se encuentran en evolución constante, incorporando herramientas o metodologías cada vez mas precisas. Básicamente se realizan siguiendo las siguientes fases:

- Evaluación de la exposición,
- Identificación del peligro,
- Caracterización del riesgo,
- Gestión del riesgo.

Las tres primeras están basadas en la aplicación de herramientas científico-técnicas, mientras que la última, la gestión del riesgo, implica una toma de decisiones considerando factores socio-económicos. En este contexto, la ecotoxicología, - rama de la toxicología que trata de entender los efectos tóxicos que los agentes físicos y químicos ejercen sobre el medio -, se ha convertido en una herramienta muy valiosa en el binomio desarrollo económico / medioambiente

La evaluación de la exposición consiste en determinar la concentración que previsiblemente puede alcanzar un xenobiótico en un determinado medio receptor.

Cuando un xenobiótico llega a un compartimento medioambiental, en función de sus características y de las del medio receptor, puede ser incorporado o absorbido por los organismos, puede formar complejos con la materia orgánica o inorgánica del medio, o puede movilizarse rápidamente a otros compartimentos (suelo - agua subterránea, etc.) Todo ello determina la concentración final que alcanzará dicho contaminante y por tanto, la magnitud de la exposición.

La evaluación de la exposición, se calcula con modelos matemáticos mas o menos complejos, considerando las *características fisicoquímicas del compuesto* -punto de fusión, solubilidad en agua, productos de degradación, persistencia, bioacumulación etc-, *su uso* -frecuencia y duración de la exposición, vías de exposición, compartimentos afectados, etc- y las *características del medio receptor* – dureza, temperatura, pH, etc-.

El resultado se conoce como Concentración Medioambiental Prevista (PEC - Predicted Environmental Concentration) y es un valor numérico que representa los niveles de exposición a los que están sometidos los organismos que viven en el compartimento estudiado.

La identificación del peligro se realiza mediante estudios de ecotoxicidad, encaminados a identificar la naturaleza de los efectos producidos por el contaminante y a establecer la relación dosis (concentración) - respuesta (efecto).

El objetivo de esta fase es estimar la **concentración** por debajo de la cual una sustancia **no** es capaz de **producir efectos adversos**. Para ello se determinan los efectos del contaminante sobre las especies representativas del compartimento estudiado, a través de todas las posibles rutas de acceso, mediante estudios de ecotoxicidad. El resultado se expresa mediante un valor numérico llamado Concentración que Previsiblemente No producirá Efectos (PNEC - Predicted No Effect Concentration)

Los ensayos de toxicidad que se requieren para la *identificación del peligro* con fines de clasificación en la Unión Europea o en la OCDE, son siempre métodos estandarizados por organismos de normalización nacionales o internacionales. Metodológicamente, los procedimientos básicos utilizados en la evaluación de la toxicidad para sustancias industriales, existentes y de nueva síntesis, biocidas etc., están recogidos en el anexo V de la directiva 67/548/EEC de clasificación, embalado y etiquetado y sus posteriores adaptaciones al progreso técnico.

En la fase de *identificación del peligro*, cuando se trata de muestras complejas, como vertidos o mezclas de contaminantes, o cuando se realiza una valoración de riesgo de un proceso industrial o cualquier otro procedimiento no enmarcado en la normativa anteriormente citada, la gama de herramientas para la valoración de efectos ecotoxicológicos se amplía considerablemente, utilizando bioensayos o “test” de ecotoxicidad mucho más versátiles y sensibles.

Un ensayo o “test” de toxicidad, en definitiva, no es mas que un procedimiento que utiliza un sistema vivo (uno o varios organismos o células) en presencia de la sustancia, compuesto o mezcla a estudiar y sobre el que se valora el efecto que produce en unas condiciones previamente fijadas. Estos resultados obtenidos en el laboratorio, deben ser capaces de predecir los efectos de la sustancia estudiada en situaciones reales. La valoración de efectos se realiza mediante un sistema escalonado. En las primeras etapas se utilizan modelos sencillos, como parámetros agudos de mortalidad (CL50) o de efecto (CE50). Posteriormente, y en función de estos resultados iniciales, se valoran efectos fisiológicos crónicos o más específicos (alteraciones en la reproducción, teratogénesis, carcinógenesis etc).

Los ensayos de ecotoxicidad pueden realizarse a cualquier nivel de organización biológica, desde moléculas hasta ecosistemas completos. No obstante, y en mayor medida que en la toxicología humana, los ensayos de laboratorio en ecotoxicología conllevan un alto grado de incertidumbre, debido a las complejas relaciones entre materia y seres vivos que existen en un ecosistema.

La elección del organismo biológico de ensayo determinará, obviamente, el valor predictivo del mismo. De esta forma, y teniendo en cuenta que existe una relación directa entre el grado de representatividad y la complejidad del sistema elegido, los estudios de campo serían los sistemas de valoración que más se asemejan a la realidad, y por tanto, con mayor valor predictivo. Sin embargo, su coste y la gran cantidad de variables a considerar,

los convierten en inapropiados, sobre todo en las primeras fases de identificación del peligro. Por esta razón solo se utilizan en ensayos confirmatorios, en casos de diagnóstico, o en aquellos casos donde el resultado de la valoración de riesgo necesite una confirmación “in situ”, considerando factores climáticos y especies autóctonas, como es el caso de algunos productos fitosanitarios.

En la práctica, el procedimiento que más se utiliza en ecotoxicología, consiste en la aplicación de una batería de ensayos agudos o subagudos utilizando especies representativas de distintos niveles tróficos para un mismo compartimento; normalmente, productores primarios, consumidores primarios y secundarios y descomponedores.

Los ensayos con especies de pequeño tamaño, (bacterias, algas o microcrustáceos) son relativamente baratos y no requieren grandes instalaciones de laboratorio. Por el contrario, la aplicación de ensayos sobre vertebrados, como los bioensayos de peces, incluso en sus estadíos más tempranos requiere de instalaciones y personal cualificado en el mantenimiento de las especies, además de un gran volumen de muestra y del sacrificio de un alto número de animales, lo que plantea problemas éticos.

Los ensayos *“in vitro”* que utilizan órganos aislados, cultivos primarios de tejidos o células derivadas de vertebrados, permiten obtener información acerca de la toxicidad de un compuesto químico o de cualquier tipo de muestra ambiental, evitando el sacrificio masivo de animales y reduciendo los

problemas técnicos y económicos. Es más, desde un punto vista mecanicista, los ensayos *"in vitro"* resultan idóneos, pues permiten abarcar un amplio numero de respuestas (mortalidad, efectos sobre el sistema inmune, teratogénicidad, mutagenicidad etc) eliminando las interferencias debidas a los efectos sistémicos.

No obstante, la aplicación de sistemas *"in vitro"* requiere un importante esfuerzo en el desarrollo del ensayo para conseguir que sus resultados adquieran precisión, sensibilidad, y lo que es más importante, que demuestren una buena correlación con el ensayo *"in vivo"* que pretenden sustituir. Todo ello es determinante para que el grado de predicción sea aceptable desde un punto de vista científico.

La caracterización del riesgo se lleva a cabo mediante la relación de los parámetros mencionados anteriormente, es decir, el nivel de exposición de un tóxico, y el efecto que produce sobre las especies seleccionadas como representativas.

La caracterización del riesgo es el resultado, por tanto, de la relación entre la PEC y la PNEC para la especie que resulte más sensible en cada compartimento ambiental estudiado.

Si el cociente PEC/PNEC es menor o igual que la unidad, se considerará que el riesgo es bajo y, por tanto, que no se requiere una mayor información toxicológica, es decir, más ensayos. Si el cociente es mayor que uno, la probabilidad de que se produzca un daño sobre el compartimiento considerado

será grande, y, por tanto, se asumirá que el compuesto tiene un alto riesgo ambiental. En ese caso se decidirá si son necesarios más ensayos, y de que tipo, o si se ha de reducir el riesgo adoptando medidas de mitigación.

La caracterización del riesgo no es una tarea fácil, ya que la vulnerabilidad de los sistemas biológicos frente a la polución por sustancias químicas depende de múltiples y complejos factores. La cuantificación de los parámetros analizados mediante ensayos ecotoxicológicos adecuados, es la única manera de realizar de forma objetiva la caracterización del riesgo. Por todo ello, es necesario incorporar metodologías cada vez más específicas y sensibles, que permitan reducir los niveles de incertidumbre que toda estimación de riesgo lleva asociada.

Por último, **la gestión del riesgo** implica una toma de decisiones tras realizar un balance coste / beneficio, considerando factores tales como la posibilidad real de control, la preocupación pública, razones políticas, éticas, intereses de los sectores de producción, mantenimiento o mejora de la calidad de vida, etc. De esta forma, la gestión del riesgo adquiere una gran importancia y sus repercusiones afectan al conjunto de la sociedad.

Un eficaz sistema de gestión medioambiental debe disponer de los conocimientos científico-técnicos necesarios para predecir el riesgo que implica la liberación de contaminantes físicos y químicos, y su valoración última debe realizarse considerando el medio ambiente como un todo.

En la actualidad, este concepto globalizador ya ha sido recogido en

algunas normativas. Así la Directiva Europea del Control y Prevención Integrado de la Contaminación (96/61/CE) (IPPC- Integrated Prevention and Control), que acaba de entrar en vigor en nuestro país, exige a los organismos responsables de la gestión medioambiental una valoración integrada de los riesgos inherentes a una determinada actividad industrial, y aunque por motivos metodológicos los análisis de riesgo se aborden por compartimentos (acuático, aéreo y terrestre), la valoración final siempre debe ser realizada conjuntamente.

1.2 MEDIO ACUÁTICO: VALORACIÓN DE EFECTOS EN POBLACIONES

Actualmente la contaminación se extiende a una buena parte de las redes hidrográficas, lagos y costas. Las causas de ello se deben en gran medida al incremento del número de industrias que vierten sus residuos, en muchas ocasiones sin depurar, al medio acuático, bien sean ríos o zonas costeras. Aunque tradicionalmente se ha tenido la tendencia a considerar que el medio acuático, por un principio de simple dilución y autodepuración, era capaz de asimilar cantidades ilimitadas de residuos; la detección de concentraciones crecientes de diferentes contaminantes químicos, metales pesados, organoclorados etc., en organismos acuáticos, ha puesto de manifiesto la poca protección que supone el principio basado en la dilución.

El compartimento acuático es sin duda el más estudiado, tanto en lo que se refiere al destino y comportamiento de los contaminantes, como en el desarrollo de metodologías para la valoración de los efectos en poblaciones acuáticas. Todo ello facilita los procedimientos de valoración de riesgo, que en éste compartimento ambiental están muy bien definidos. De hecho, el interés

científico en la actualidad se ha desplazado desde la valoración de los efectos agudos, a considerar los efectos transgeneracionales que ejercen ciertos compuestos químicos sobre las poblaciones naturales. (Colborn et al., 1996; Anderson et al., 1994).

Estos efectos pueden manifestarse de forma indirecta dando lugar a un decrecimiento de la supervivencia, de la tasa de reproducción, edad de maduración etc., de los individuos pertenecientes a las poblaciones afectadas. De esta manera se provoca un proceso de selección o cuello de botella dirigido por la acción del tóxico (Theodorakis et al., 2001) En consecuencia, la diversidad genética puede verse disminuida, e incidir negativamente sobre la capacidad de adaptación, viabilidad y persistencia de la población expuesta. (Bickham and Smolen, 1994; Bickham et al., 2000) (Figura 1)

También los efectos transgeneracionales pueden manifestarse de manera directa por la acción de los denominados agentes genotóxicos, contaminantes capaces de interaccionar y modificar la molécula de ADN. La alteración del ADN de las células somáticas incrementa la incidencia de determinados tipos de tumores, mientras que la alteración del ADN de las células germinales de los individuos expuestos se transmite a la descendencia. De esta forma, las alteraciones a escala celular pueden modificar, finalmente, y tras periodos más o menos largos de tiempo, la estructura genética de estas poblaciones (Bickham et al., 2000) (Figura 2)

Las especies piscícolas están directamente afectadas por ambos procesos, pero los estudios a escala poblacional plantean graves problemas prácticos, como disponer de poblaciones control, e incluso de metodologías sensibles y específicas que permitan predecir de manera inequívoca, si un contaminante es capaz de producir alteraciones en la dotación genética en una población. Sin embargo son numerosos los trabajos que asocian la presencia de neoplasias en una gran variedad de especies piscícolas, con la presencia en el agua de xenobióticos. (Harshbarger and Clark, 1990; Leblanc and Bain., 1997; Malins et al., 1996; Dawe, 1969; Al-Sabti and Metcalfe, 1995) Por tanto, y aunque en ecotoxicología el centro de interés se establece a escala poblacional mas que individual, la posibilidad de detectar "a priori" alteraciones genéticas en células somáticas adquiere una gran importancia, permitiendo identificar compuestos de relevancia ambiental (Shugart, 2000; Weinstein, 1988).

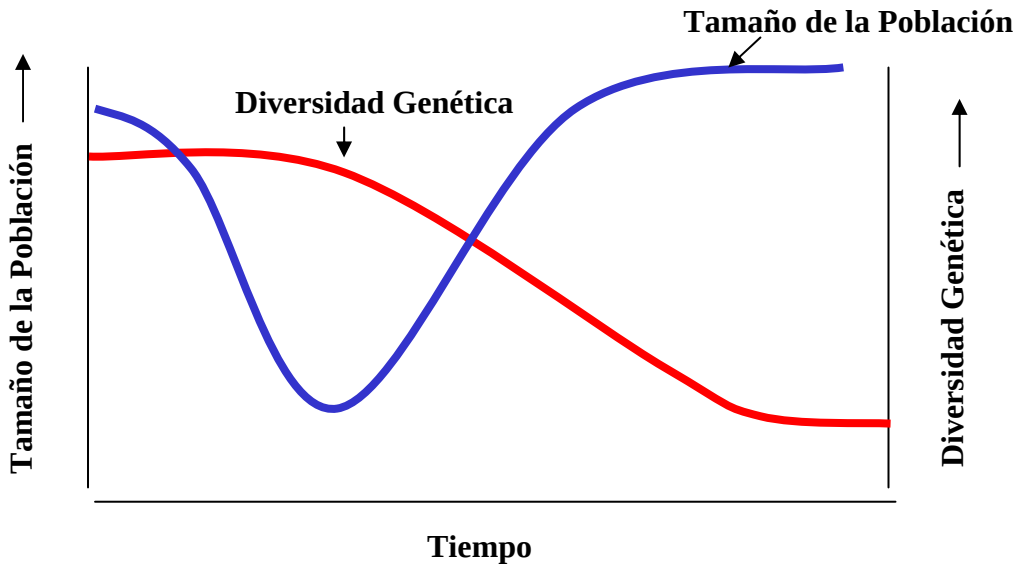

Figura 1. Relación entre diversidad genética y tamaño de la población. (Bickham et al., 2000)

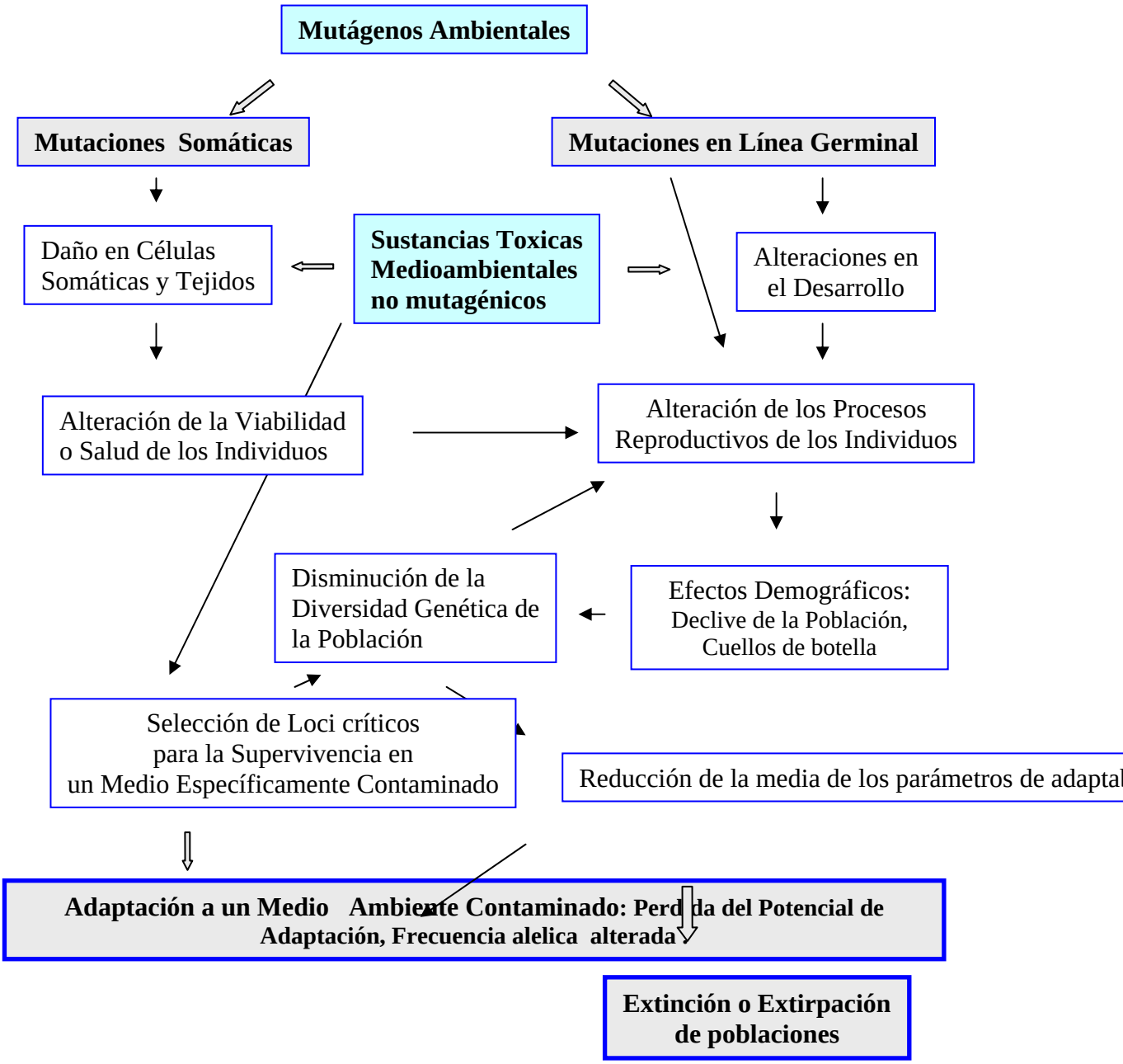

Figura 2. Relación entre los distintos efectos causados por la contaminación ambiental (compuestos mutagénicos o no mutagénicos) y la pérdida de diversidad biológica. (Bickham et al., 2000)

En este sentido, y al igual que en los estudios de genotoxicidad en mamíferos, resultan de gran utilidad los ensayos "*in vitro*". La utilización de líneas celulares derivadas de especies piscícolas representativas constituye una alternativa real a los bioensayos de peces, y presenta grandes ventajas técnicas como, por ejemplo, la capacidad de abordar un gran numero de muestras con instalaciones de laboratorio poco sofisticadas, lo que se traduce en una reducción de los costes. En el ámbito metodológico, la homogeneidad genética de las células y su ciclo celular, permite simular estudios transgeneracionales en periodos relativamente cortos de tiempo y con volúmenes de muestra reducidos (Tabla I) (Rachlin and Perlmuter 1968, Babich and Borenfreund 1991; Castaño et al., 1994; 1996; 2000; Segner, 1998, Fent 2001).

Concretamente, la línea celular RTG-2 derivada de tejido gonadal de trucha arcoiris (*Oncorhynchus mykiss*), es una de las mejor caracterizadas (Figura 3) Se estableció a principios de los años sesenta por Wolf y Kimby y está incluida en la colección de cultivos celulares americana (ATCC) y europea (ETCC). Es una línea de morfología fibroblástica, con una dotación cromosómica de 2n = 60, que retiene capacidad para metabolizar xenobióticos (Clark, and Diamond, 1970; Thornton, et al., 1982) y que es viable en un amplio rango de temperaturas (4 a 23º C), lo que permite investigar múltiples variables cinéticas, y además resulta extremadamente útil en la práctica diaria de laboratorio.

ENSAYO	COMPUESTO	LÍNEA ESTABLECIDA	CULTIVO PRIMARIO	BIBLIOGRAFÍA
Aductos de ADN	B(a)P, DMBA	BF-2, RTG-2, BB		Smolarek et al, 1987, 1988
	Aflatoxina B1		Hepatocitos de trucha arcoiris	Bailey et al, 1982
	B(a)P		Hepatocitos de trucha arcoiris	Masfaraud et al, 1992
Alkaline Unwinding Assay	MND, PQ	BB		Hasspieler et al, 1996
Ensayo Cometa	MNNG, H_2O_2	R1, RTG-2		Braunbeck and Neumüller, 1996
	Extractos orgánicos de sedimentos marinos	EPC		Kamman et al, 2000; 2001
	Cadmio		Hepatocitos de trucha	Risso-de Faverney et al, 2001
	B(a)P, 4NQO	RTG-2, RTL-W1		Nehls and Segner, 1998
	Muestras ambientales	RTG-2, RTL-W1		Nehls and Segner, 2001
	Muestras de agua		Hepatocitos de pez cebra	Schnurstein and Braunbeck, 2001
	Muestras de agua		Pez cebra células branquiales	Schnurstein and Braunbeck, 2001
	H_2O_2, B(a)P		Hepatocitos de trucha arcoiris	Devaux et al, 1997
	H_2O_2, furanona, B(a)P, 1-nitropireno		Hepatocitos/sangre trucha	Mitchelmore et al, 1998
	H_2O_2		Células rojas Pleuronectes	Nacci et al, 1996
	Metil mercurio		Linfocitos de T. Truncatus	Betti et al, 1996
	Ciclofosfamida		Eritrocitos carpa	Pandrangi et al, 1995

ENSAYO	COMPUESTO	LÍNEA ESTABLECIDA	CULTIVO PRIMARIO	BIBLIOGRAFÍA
Aberraciones Cromosómicas (AC)	B(a)P, MNNG, MMC, AA, 3-MC	RTG-2		Kocan et al, 1982, 1985 Kocan and Powell, 1985
	Sedimentos marinos	RTG-2		Kocan et al, 1985 Landolt and Kocan, 1984 Ahne and Schweitzer, 1993
	Atrazina, meturxoron,4-ClA, HCH, PCP,alacloro,carbofurano	R1		Ahne and Schweitzer, 1993
	N-nitroso-N-methylurea	ULF-23		Park et al, 1989
	3,4-BP, 1,2,5,6-DBA, 1,2-BA, pyrene (PY).	Ul-h		Walton et al, 1988
Intercambio de Cromatidas Hermanas (SCE	MMC, MNNG, MMS	Línea celular A. splendens		Barker and Rackham, 1979
	MNNG	ULF-23		Suyamah and Etoh, 1988
	N-nitroso-N-methylurea	ULF-23		Park et al, 1989
			Leucocitos en cultivo	Ellingham et al, 1986
			Leucocitos en cultivo	Zakour et al, 1984
	EMS, MMS		Leucocitos	Maddock and Kelly, 1980
Inducción de Micronúcleos	Fenil-, etil-, metil-organomercuriados	BG/F		Babich et al, 1990
	MNNG, 4NQO	Ul-h		Walton et al, 1984
	B(a)P, DP, EMS	RTG-2		Kolpoth et al, 1999
	MMC, B(a)P, VS	RTG-2		Sanchez et al, 2000
	Mezclas complejas	RTG-2		Castaño et al, 2000
Síntesis no programada de ADN (UDS)	MNNG, 4NQO, aflatoxin B1	RTG-2		Walton et al, 1983
	MNNG, 4NQO	Ul-h		Walton et al, 1984
	B(a)P, Aflatoxina B1	RTG-2 + S9		Walton et al, 1987
	Sedimentos	BB		Ali et al, 1993
			Hepatocitos de peces	Klaunig, 1984
			Hepatocitos de peces	Kelly and Maddock, 1985
			Hepatocitos de peces	Miller et al, 1989
	Irradiación UV	CAF-MM1		Mano et al, 1980
	Irradiación Gamma	CAF-MM1		Mitani et al, 1982 Mitani and Egami, 1982

Esta línea celular se ha utilizado para la valoración de la toxicidad aguda de compuestos representativos de casi la totalidad de los grupos de contaminantes ambientales, mostrando una muy buena correlación con los resultados obtenidos “in vivo” (Babich and Borenfreund, 1991; Segner 1998; Castaño et al., 1996; 2000), en estudios citogenéticos (Kohlpott, et al., 1997; Sánchez et al., 2000) así como, más recientemente, para la detección de compuestos que modifican el comportamiento hormonal o disruptores endocrinos (Fent, 2001)

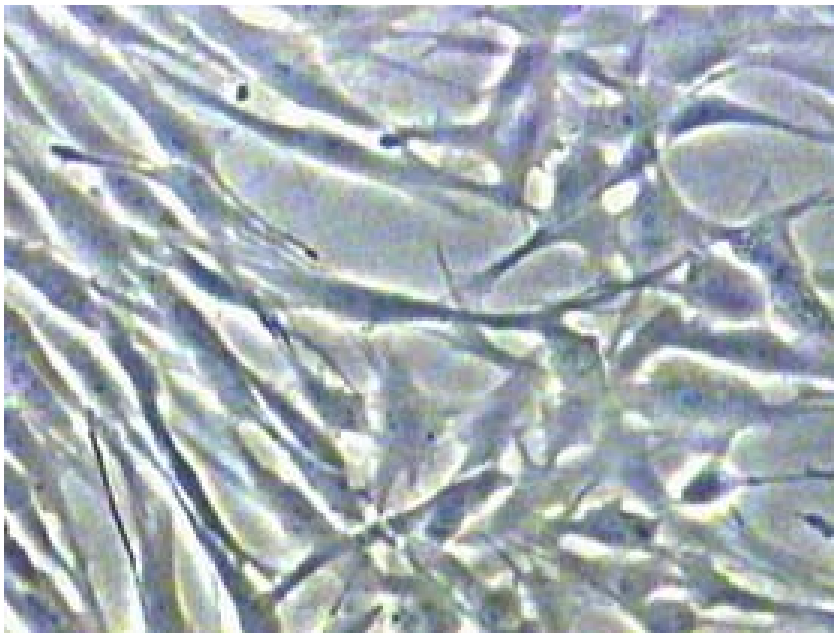

Figura 3. Línea celular RTG-2 derivada de tejido gonadal de trucha arcoiris (*Oncorhynchus mykiss*),

En los estudios de genotoxicidad, los sistemas “in vitro” constituyen el primer paso para la identificación de efectos. Una vez que el mutágeno interacciona con la molécula de ADN, se producen en ella, tras cortos periodos

de tiempo (minutos e incluso segundos), daños estructurales cuya naturaleza está en función de las características físico-químicas del compuesto (Tabla II)

Existe un importante grupo de sustancias con capacidad genotóxica (algunas de ellas tras ser metabolizadas), que forman enlaces covalentes con la molécula de ADN, dando lugar a los llamados aductos (Figura 4). En los últimos años se han desarrollado diferentes metodologías capaces de cuantificarlos, aplicando técnicas inmunológicas con anticuerpos específicos (Poirier, 1984; Santella, 1988), HPLC combinado con espectroscopia fluorescente (Dunn, 1991; Krahn et al., 1993), cromatografía de gases acoplada a espectrometría de masas (Krahn et al., 1993) o marcaje con ^{32}P también llamado "^{32}P-postlabeling".

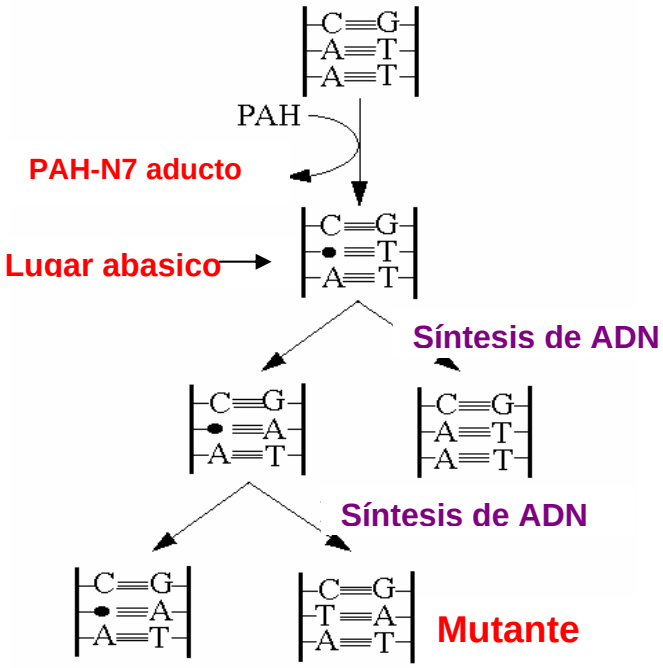

Figura 4. Formación de mutaciones a partir del aducto formado entre un hidrocarburo policíclico aromático (PAH) y el N - 7 de la adenina.

Esta última, es quizás la más utilizada debido a su gran especificidad y sensibilidad ya que es capaz de detectar un aducto entre 10^{10} nucleótidos normales. (Gupta et al., 1982; 1985; 1988; Liu et al., 1988; Halbrook et al., 1992; Lloyd-Jones, 1995; Jones and Parry, 1992; Pfau 1997; Qu et al., 1997)

La formación de aductos, está considerada como un elemento clave en la iniciación de procesos carcinogénicos, ya que pueden desestabilizar las uniones glucosídicas y dar lugar a lugares apurínicos con posterior rotura de la cadena de ADN, o pueden dar lugar a la inserción de bases incorrectas, que tras el proceso de replicación pueden comprometer la fidelidad de la copia. (Weinstein, 1978; Wogan and Goreling, 1985).

Los daños sobre el ADN, además de por la formación de aductos, pueden producirse por otros procesos diferentes, como son la generación de especies de oxígeno altamente reactivas, (Kuchino et al., 1987; White et al., 1996; Park et al., 1996; Sugg et al., 1996) o dando lugar a mutaciones por formación de dímeros de pirimidina, desaminación de bases, o interfiriendo con los procesos de replicación, metilación o reparación (Shugart, 2000). La hipometilación, consecuencia, por ejemplo, de la oxidación de las bases por radicales libres, puede medirse mediante la hidrólisis enzimática del ADN (Rosiello et al., 1994; Count and Goodman ,1995) o mediante la síntesis no programada de ADN (Selden et al., 1994).

En cuanto a la detección de capacidad mutagénica la, los ensayos bacterianos tradicionales de mutagenicidad, como el “test” de Ames (Ames et

al., 1975; Stepanova et al., 1999), están siendo sustituidos por ensayos de activación de oncogenes (Deveraux, 1994; Abshire et al., 1996), o la utilización de modelos con animales transgénicos (Jowett et al., 1991; Murti et al., 1994; Ryskova et al., 1997)

La detección de roturas en las cadenas de ADN puede realizarse mediante los ensayos de desnaturalización en medio alcalino, (Shugart, 1988; 1998; 1996b; Meyers-Schone et al., 1993; Everaart et al., 1998), electroforesis en geles alcalinos o neutros (Theodorakis et al., 1994) o el ensayo Comet (Ostling and Johanson 1984; Michelmore and Chipman 1998; Singh et al., 1988; Olive et al., 1992; Fairbairn et al., 1995), todos ellos basados en la desnaturalización “in vitro” de la doble hélice a determinado pH. La proporción de ADN de cadena sencilla detectada se considera que está relacionada con el número de roturas.

Los daños estructurales son reparados la mayor parte de las veces por los mecanismos celulares, pero cuando no es así y persisten o son reparados erróneamente, pueden interferir en la fidelidad de la replicación y producir cambios irreversibles en el genoma. Estos efectos tardíos e irreversibles, tales como el intercambio de cromátidas, las aberraciones cromosómicas o el incremento en la frecuencia de micronúcleos, se denominan efectos citogenéticos y para la detección de los mismos se han incorporado recientemente técnicas de análisis automático de imagen y de citometría de flujo. (Gordon et al., 1989; Custer et al., 1994; 1997; Lamb et al., 1995; Grawe

et al., 1997; Bikham et al., 1998; Carrano et al., 1978; Kohlpoth et al., 1999; Sánchez et al., 2000)

En función de la magnitud del daño producido al ADN, sus efectos pueden no ser fácilmente observables, siendo necesarios meses o años para hacerse patentes, a veces incluso, después de que la fuente de contaminación haya sido eliminada. Son precisamente estos eventos tardíos, los que a escala poblacional adquieren mayor importancia (Saava, 1998; Anderson et al., 1994) (Tabla II) Por tanto, el desarrollo de metodologías capaces de detectar el daño al ADN en estadíos tempranos adquiere una gran importancia.

Tabla II. Secuencia de efectos de la exposición a genotóxicos.

Efectos biológicos al nivel de individuo			**Efectos a escala poblacional**
Temprano	**Temprano-Medio-Tarde**	**Medio /Tarde**	**Tarde**
1. *Detoxificación* • Inducción del sistema enzimático P_{450} **2.** *Alteraciones estructurales* Agentes físicos • Dímeros de Timidina • Rotura de cadena Agentes químicos • Aductos • Alteraciones de bases • Rotura de cadenas • Modificación de bases • Hipometilación • Interferencias con la reparación y replicación del ADN • Mutaciones	**3.** *ADN anormal* • Aberraciones cromosómicas. • Micronúcleos • Aneuploidías • Apoptosis	**4**. *Condiciones patológicas* • Neoplasias • Tumores • Disfunción de proteínas • Declive de la fertilidad • Letalidad	**5.** *Efectos genéticos* • Alteración de la diversidad genotípica • Variaciones en la frecuencia alélica **6.** *Efectos fenotípicos* • Rasgos fenotípicos alterados. • Modificaciones en la estructura de las clases de edad. • Decrecimiento de la abundancia y distribución de la población • Extinción de la población

El tiempo de aparición de estos efectos dependerá de las especies y tipo de genotóxico. (**Temprano**: horas o días. **Medio**: días, semanas o meses. **Tarde**: semanas, meses o años.) Modificada a partir de Thomson et al., 1999

1.3 ANTECEDENTES METODOLÓGICOS DE LA TÉCNICA DE RAPD

En la última década, los avances en biología molecular han permitido el desarrollo de técnicas tales como el análisis de polimorfismos obtenidos mediante enzimas de restricción (RFLP), y la amplificación de genes mediante la Reacción en Cadena de la Polimerasa (PCR), abriendo nuevas posibilidades en la detección del daño al ADN. (Botstein et al., 1980; Saiki et al., 1988; Nirmal et al., 1999)

La técnica de PCR, fue desarrollada por Mullis a finales de los años ochenta y con ella se consigue la amplificación enzimática de un fragmento de ADN o ARN hasta millones de veces, de un modo rápido y fiable. Su gran sensibilidad, sencillez y eficacia para amplificar específicamente determinados segmentos de ácidos nucleicos, ha permitido afrontar problemas experimentales que antes eran irresolubles. En ella se imita, bajo condiciones controladas de laboratorio, el proceso de replicación del ADN que tiene lugar en los organismos vivos (Figura 6)

La complejidad del ciclo biológico, se simplifica *"in vitro"* utilizando una ADN polimerasa y ciclos de temperatura para desnaturalizar e hibridar alternativamente, la parte de ADN que se desee obtener. El resultado es la amplificación exponencial de determinados fragmentos de ADN seleccionados mediante cebadores o "primers" diseñados por el analista. El principal inconveniente de la PCR, es que requiere el conocimiento previo de las

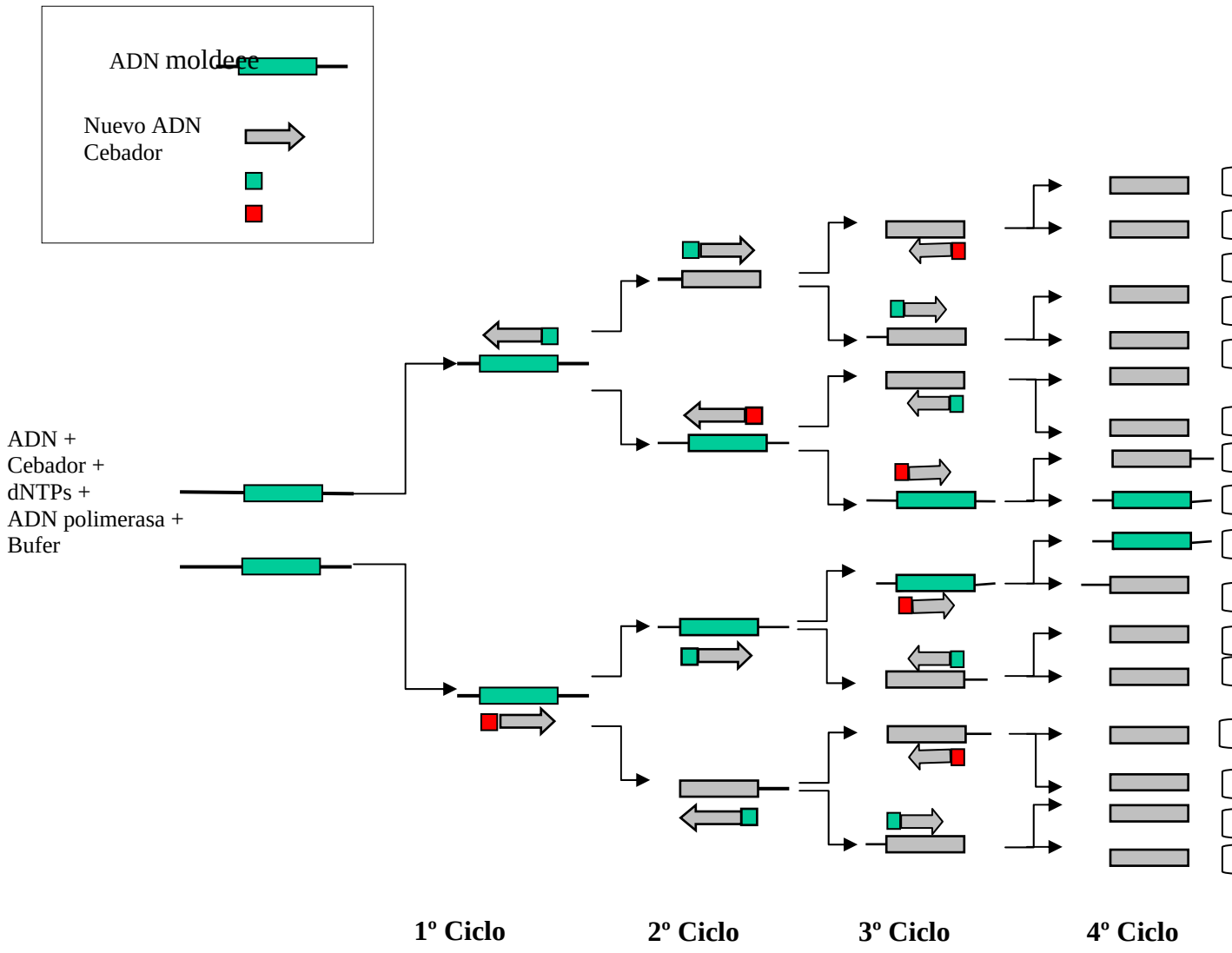

Figura 6. Esquema de la técnica de PCR. Después de 30 ciclos se obtienen 1 billón de copias idénticas de un fragmento determinado de ADN.

secuencias que flanquean el fragmento a amplificar, cosa que no siempre es posible.

Para solventar este problema se desarrollaron una serie de variantes conocidas con el nombre de MAAP (Multiple Arbitrary Amplificon Profiling o Técnicas de Obtención de Perfiles Múltiples y Arbitrarios de Amplificación), que utilizan cebadores de secuencia arbitraria y generan una huella genética del ADN molde estudiado. Este grupo está formado por la técnica de RAPD (Random Amplified Polimorphic ADN o ADN Polimórfico Amplificado al Azar) (Williams et al., 1990), AP-PCR (Arbitrarily Primed PCR o Reacción en Cadena de la Polimerasa Usando Cebadores al Azar) (Welsh and McClelland 1990) y DAF (ADN Amplification Fingerprinting o Huella Genética Mediante Amplificación del ADN) (Caetano-Anolles et al., 1991) Las diferencias entre ellas, consisten en la longitud del cebador utilizado y en la forma de visualizar los fragmentos amplificados (Tabla III)

La técnica de RAPD, desarrollada por Williams y col., en 1990 utiliza cebadores cortos (entre 9-14 bases) de secuencia arbitraria, y la reacción de amplificación se realiza a una temperatura de hibridación o “anillamiento” poco selectiva, alrededor de 36ºC. Con estas condiciones la especificidad del cebador disminuye, aumentando la probabilidad de hibridación, aunque en muchos casos de forma imperfecta. Las diferencias entre PCR y RAPD aparecen en la tabla IV.

Tabla III. Principales diferencias entre las Técnicas de Obtención de Perfiles Múltiples y Arbitrarios de Amplificación.

	RAPD	AP-PCR	DAF
• Cebadores	10-12 pb	20 pb	5 pb
• Ciclo de temperaturas	Baja tª de hibridación en todos sus ciclos (30-39ºC)	Baja tª de hibridación en los primeros 2-5 ciclos alta en el resto (45-50ºC)	Baja tª de hibridación en todos sus ciclos (30ºC)
• Nº de ciclos	40-45	40-45	30-35
• Visualización de los productos	Electroforesis en geles de agarosa + bromuro de etídio	Incorporación de α^{32}P-dATP en los primeros ciclos. Electroforesis en geles de poliacrilamida + autoradiografía	Electroforesis en poliacrilamida + tinción con plata
• Tamaño de los productos	Entre 300 y 2000 pb	Por encima de 500 pb	Por encima de 500 pb

(pb = pares de bases) Datos tomados de Menier y Grimont (1993)

Tabla IV. Diferencias básicas entre la técnica de PCR y RAPD.

PCR	RAPD
➢ Secuencia del cebador complementaria al DNA molde conocido.	➢ Secuencia del cebador al azar, no requiere del conocimiento del ADN molde.
➢ En ausencia de la secuencia no se produce amplificación.	➢ Por lo general, se encuentran secuencias complementarias.
➢ No exige integridad al ADN molde.	➢ Es fundamental la integridad del ADN molde
➢ Ciclo de temperaturas (tª de hibridación: 50 - 55ºC)	➢ Ciclo de temperaturas (tª de hibridación : 30 - 39ºC)
➢ Nº de ciclos: 30	➢ Nº de ciclos: 45

Durante los primeros ciclos el proceso es competitivo, es decir, inicialmente un numero elevado de fragmentos son co-amplificados, pero solo aquellos que presentan una mayor estabilidad cebador-ADN molde serán los productos finales de la reacción.

El resultado, es un amplio espectro de fragmentos amplificados que conforman un patrón de bandas o huella genética característica del ADN molde utilizado. Los fragmentos o bandas, generalmente entre 300 y 2000 pares de bases, son separados electroforeticamente en geles de agarosa y visualizados mediante una tinción con bromuro de etídio. La técnica presenta grandes ventajas ya que:

- No necesita conocimiento previo de la secuencia a amplificar.
- Utiliza pequeñas cantidades de ADN molde (entre 5-50 ng)
- Utiliza los mismos cebadores para distintos sistemas biológicos.
- Puede analizar un número elevado de muestras en poco tiempo.
- No necesita marcaje radiactivo ni grandes equipos de laboratorio.

Todas ellas, han hecho que esta técnica se aplique con éxito en una gran variedad de campos: en *epidemiología molecular* para la identificación de cepas patógenas causantes de infecciones bacterianas (Allerberger et al., 1997; De Vos et al., 1997), en el *sector alimentario* permitiendo la detección de brotes de intoxicación (Loudon, et al., 1996) o el seguimiento de las vías de diseminación de patógenos durante el procesamiento de alimentos

(Niederhauser, et al., 1994); en estudios taxonómicos estableciendo relaciones de proximidad evolutiva en especies animales y vegetales (Rothmanier, et al., 1997; Rout et al. 1998; Hoey et al., 1996); en la elaboración de mapas genéticos (Williams, et al., 1993), etc.

También, aunque más tímidamente, se ha utilizado en estudios de genotoxicidad humana y animal. Peinado y colaboradores la aplicaron para la detección de mutaciones en células tumorales de colon, tras comparar sus patrones de bandas con los de las células sanas (Peinado et al., 1992). En ecotoxicología, Kubota y colaboradores irradiaron machos del pez "medaka" (*Oryzias latipes*), con el objetivo de detectar alteraciones en el ADN de los individuos irradiados y en su progenie. Los resultados confirmaron la alteración del patrón mediante la perdida y ganancia de bandas, así como diferencias en la intensidad de amplificación. Verificaron el carácter hereditario de alguna de las bandas de nueva aparición, sin que ello afectara a la viabilidad de la progenie (Kubota et al., 1995). Además, demostraron una relación directa entre las alteraciones en el patrón y la intensidad de irradiación (Kubota et al., 1992).

No obstante, esta técnica ha sido criticada por la aparición de bandas erráticas de difícil interpretación y, en general, por la falta de reproducibilidad de sus resultados, aspectos muy estudiados aunque no bien entendidos.

La naturaleza e intensidad de las bandas, está en función de múltiples parámetros, tales como la integridad del ADN molde utilizado y/o de cada una de las condiciones que intervienen en la reacción, desde la elección de la ADN

polimerasa hasta el ciclo de temperaturas establecido. (Davin-Regli et al., 1995; Abed et al., 1995; Bassam et al., 1992; Ellsmorth et al., 1993; Meunir and Grimont 1993; Park and Kohel, 1994; Penner et al., 1993; Williams et al., 1990).

A su vez, la integridad del ADN molde está en función de las condiciones iniciales de la muestra, del método de extracción utilizado, así como de la conservación posterior del extracto genómico obtenido. (Black, 1993; Cheng et al., 1995). En cuanto a la unión cebador–ADN molde, es directamente dependiente de la temperatura de hibridación y pequeñas variaciones en ella pueden modificar los lugares de anillamiento y, por tanto, los productos finalmente amplificados.

2. JUSTIFICACIÓN Y OBJETIVOS

La presencia continuada de sustancias químicas de naturaleza genotóxica, provoca daños estructurales en el material hereditario de las poblaciones piscícolas expuestas. La posibilidad de evaluarlo tempranamente, representa un avance metodológico en los procedimientos de valoración de riesgo.

En los últimos años, y gracias al incipiente auge de la genómica, se han desarrollado técnicas sensibles y específicas que abren nuevas posibilidades en disciplinas científicas muy dispares.

Aún así, la aplicación de dichas metodologías en estudios poblacionales de peces, plantea dificultades técnicas, económicas y éticas, debido al alto número de animales, a las instalaciones de laboratorio que requieren y a la larga duración de los estudios. Estas dificultades pueden en parte solventarse mediante la utilización de sistemas *"in vitro"*, particularmente en las primeras fases de valoración de riesgo.

Nuestros estudios han ido dirigidos a conseguir un modelo sencillo y suficientemente representativo del medio acuático, que permita detectar una amplia gama de alteraciones en la molécula de ADN, asociadas a la acción de sustancias genotóxicas medioambientalmente relevantes.

El sistema biológico elegido ha sido la línea celular RTG-2, derivada de

trucha arcoiris, y, que como se ha mencionado anteriormente, ha presentado una buena correlación en multitud de estudios de citotoxicidad con el sistema *"in vivo"* del que procede.

La técnica de RAPD, de reciente desarrollo, ha sido elegida para la detección de efectos sobre dicho sistema. Teniendo en cuenta su fundamento teórico, es posible detectar alteraciones inespecíficas en el ADN procedente de células que posean una dotación genética idéntica, como es el caso de las líneas celulares establecidas, mediante la comparación del patrón de bandas de las células expuestas a la acción de genotóxicos y el patrón de bandas de células no expuestas o controles. El hecho de no requerir información de la secuencia genómica representa una gran ventaja en estudios ecotoxicológicos, donde el conocimiento de la genética de las especies silvestres es muy reducido, y donde el interés se centra en la detección del daño, y sus posibles consecuencias, más que en la naturaleza del daño en sí mismo, objeto de otras disciplinas como la genética molecular o la bioquímica.

Además de lo anterior, la técnica de RAPD es simple y no necesita marcaje radiactivo, lo que permite que pueda realizarse prácticamente en cualquier laboratorio. Por lo tanto, considerando la gran cantidad de ventajas que presenta teóricamente esta técnica en estudios de ecotoxicología genética, y aunque ha sido criticada por la falta de reproducibilidad de sus resultados, nos hemos planteado esta tesis marcándonos dos objetivos concretos:

1. Conseguir resultados fiables y reproducibles, optimizando todos y cada uno de los diferentes parámetros que intervienen en la reacción RAPD.

2. Desarrollar un sistema *"in vitro"* sensible y versátil, que permita valorar a priori el carácter genotóxico de compuestos químicos y muestras ambientales.

3. PLANTEAMIENTO Y RESULTADOS

El desarrollo metodológico ha ido dirigido a conseguir un sistema sensible y cuyos resultados *"in vitro"* sean capaces de predecir los resultados *"in vivo"*. Como las variaciones, incluso muy pequeñas, en el patrón de bandas de células control y células expuestas son asumidas como resultado de la acción del tóxico sobre el ADN, es imprescindible que dicho patrón sea específico y altamente reproducible.

Por tanto, este trabajo se ha estructurado de la siguiente manera:

1. **OPTIMIZACIÓN DE LA TÉCNICA (Reproducibilidad y Especificidad)**
2. **CAPACIDAD PARA LA DETECCIÓN DE DAÑO AL ADN (Sensibilidad)**
3. **CAPACIDAD PARA PREDECIR EL DAÑO *"IN VIVO"***

3.1 OPTIMIZACIÓN DE LA TÉCNICA. (Reproducibilidad y Especificidad) (Anexos 2.1 y 2.4)

Como ya se ha mencionado, uno de los aspectos mas criticados a la técnica de RAPD, ha sido la falta de reproducibilidad de sus resultados, dando lugar a patrones de bandas variables. Los factores que intervienen en esta falta de reproducibilidad se pueden agrupar en:

1. **Integridad del ADN.**
2. **Ajuste de los Parámetros de la Reacción.**

3.1.1. La Integridad del ADN, a su vez, está determinada por el método de extracción **seleccionado y por el** estado de conservación de los extractos**.**

Para seleccionar **el método de extracción** mas adecuado, se ensayaron tres técnicas diferentes: (Anexo 2.1)

- *Precipitación de proteínas* en un medio con alto contenido en sales (Salting out) (Miller et al., 1988; Cheng et al., 1995).
- *Ebullición en presencia de "Chelex"* como resina quelante de metales divalentes. (Walsh et al., 1991).
- *Extracción con Fenol-cloroformo* basado en la extracción diferencial, de la molécula de ADN (Sambrook et al., 1988).

La idoneidad del método de extracción se valoró analizando la **concentración, pureza** e **integridad** de cada uno de los extractos geonómicos**.**

La concentración se determinó midiendo la absorbancia que presenta a 260 nm; y **la pureza,** mediante la relación de absorbancias a 260/280 nm. Adicionalmente se introdujo una medida a 320 nm para sustraer el fondo

inespecífico, tras comprobar la distorsión que éste producía en un estudio realizado con 18 extractos genómicos.

La integridad se determinó realizando una electroforesis en gel de agarosa (al 0,8%) de una alícuota del extracto. La presencia en el gel de una sola banda de aproximadamente 23.000 Kb indica el buen estado del ADN extraído. En caso contrario, se observa la presencia de varias bandas de pesos moleculares significativamente mas bajos, o bien una fluorescencia difusa a lo largo de toda la calle.

Los resultados mostraron que el método más adecuado para la técnica de RAPD era la extracción con **Fenol-Cloroformo**.

La conservación de los extractos de ADN (anexo 2.4) es determinante para mantener el tamaño y el número de fragmentos en el patrón de amplificación.

Generalmente, bien por conveniencia o por el propio diseño del ensayo, el proceso de extracción del ADN se realiza simultáneamente en un número elevado de muestras, almacenando los extractos hasta el momento de su análisis, en algunos casos después de semanas o meses. En muchas ocasiones, el almacenamiento conlleva un deterioro del extracto y, por tanto es fundamental determinar los márgenes dentro de los cuales se puede realizar un ensayo en condiciones de seguridad.

Se valoraron, a intervalos regulares de 3, 6, 9, 15 y 21 meses, la integridad y concentración del ADN de 18 extractos genómicos almacenados a 4º C durante un periodo de casi 2 años.

Los resultados mostraron que los extractos pueden ser utilizados durante un periodo máximo de 9 meses. Pasado este tiempo, y aunque la concentración de ADN permanece prácticamente constante, su integridad se ve alterada significativamente.

3.1.2. Ajuste de los parámetros de la reacción. (Anexo 2.1)

Con respecto a la enzima el Fragmento Stoffel de la Taq polimerasa, existe acuerdo unánime en que esta enzima, obtenida a partir de la Taq polimerasa por modificación genética, mejora la reproducibilidad en la reacción, y, aunque no se conoce la causa concreta, se apunta a una mayor estabilidad térmica, menor velocidad de procesamiento (10 veces menor que la Taq polimerasa) y / o el amplio rango de iones magnesio (2 -10 mM) en el cual trabaja. (Lawyer et al., 1988; 1993). El resto de los componentes de la reacción, aunque se estudiaron diferentes concentraciones de Mg y dNTPs, se fijaron finalmente dentro de los rangos recomendados: 4 mM de Mg y 0,2 mM de cada dNTPs y 2 U del enzima.

En relación a las **concentraciones optimas de ADN y primer** utilizadas en la reacción, existen opiniones contradictorias. Nosotros hemos estudiado un amplio rango de concentraciones, desde 1 a 100 ng/25 ul de ADN y desde 1 a 8,5 pM de primer. Los resultados nos indujeron a elegir, inicialmente, 15 ng/25

ul y 5 pM de ADN y primer respectivamente, como las proporciones mas adecuadas. Se observó que cada cebador, en función del número de bandas que amplifica, presenta sus propias concentraciones óptimas, lo que nos llevaría a ajustar las concentraciones en futuros trabajos.

La unión cebador–ADN molde es directamente dependiente de la **temperatura** de hibridación, y pequeñas variaciones en ella pueden modificar los lugares de unión y, por tanto, los productos finales amplificados. (Penner et al., 1993; Roehrdanz et al., 1993). En éste sentido se estudiaron, un rango de temperaturas de hibridación (entre 34º a 39º C), **tiempos** y **numero de ciclos**. Los resultados obtenidos permitieron establecer la temperatura idónea en **36º C durante 75 seg. y 50 ciclos**.

En lo referente a la **visualización de los fragmentos** amplificados, ésta se realiza mediante electroforesis en geles de agarosa y tinción con bromuro de etídio. Tras estudiar diferentes voltajes, tiempos (de 1 a 4h) y concentraciones, se determinó una tensión de 115 V durante 4 h y 2.1% de agarosa como condiciones óptimas para conseguir una buena separación entre los fragmentos o bandas. La incorporación del bromuro de etídio (0,5 µg/ml) se realiza durante la preparación del gel, antes de que se solidifique, lo que permite la comparación fluorimétrica de las bandas, a la vez que se minimiza el manejo de este compuesto.

Para evitar la subjetividad en la interpretación de los resultados por parte del operador, la imagen del gel es capturada y analizada por densitometría

mediante soporte informático.

3.2. CAPACIDAD PARA LA DETECCIÓN DE DAÑO AL ADN (Sensibilidad) (Anexos 2.1, 2.2, 2.4, 3.1, 3.2)

La detección de alteraciones en el ADN de las células expuestas es teóricamente sencilla, ya que las variaciones observadas en los patrones de bandas de células controles y expuestas son interpretadas como resultado de la acción del compuesto genotóxico sobre el ADN. Por ello es importante obtener:

1. **Un Patrón de Bandas Específico y Reproducible.**
2. **Unas Condiciones Óptimas para Detección de Daño al ADN.**
3. **Un Sistema de Análisis** suficientemente sensible para detectar alteraciones en el patrón de bandas.

3.2.1 Patrón de Bandas Específico y Reproducible. (Anexo 2.1, 2.2)

Una vez fijadas las condiciones de la reacción, dicho patrón de bandas se consiguió mediante una adecuada selección de primers.

Se probaron 26 primers. Algunos no generaron bandas, otros proporcionaron productos de amplificación difusa o bandas poco definidas y en otros casos, aunque se obtuvieron bandas definidas, no presentaban reproducibilidad intra y/o inter extractos, por lo que se seleccionaron solo aquellos que presentaban bandas claras y altamente reproducibles. También

se estudiaron combinaciones binarias de cebadores, ninguna de las cuales presentó resultados satisfactorios, bien por fallos en la amplificación, bien por pérdida de reproducibilidad intergenómica, o bien por la aparición de bandas con iguales pesos moleculares que los utilizados individualmente, por lo que la combinación de primers no aportaba ninguna ventaja.

Finalmente, la huella genética de las células pertenecientes a la línea celular RTG-2 quedó establecida con 8 cebadores que generaban un total de 56 bandas, con una reproducibilidad superior al 80% en todas las amplificaciones y dentro de un amplio rango de pesos moleculares (aproximadamente entre 2000 y 300 pares de bases)

La reproducibilidad de los patrones se comprobó amplificando extractos procedentes de diferentes pases celulares (entre el pase 100 y el 135).

La **especificidad** de la huella obtenida se constató comparando los patrones de bandas de otras líneas celulares derivadas de carpa (EPC) y de hámster (CHO-K1)

3.2.2 Condiciones Óptimas para la Detección de Daño al ADN.

(Anexos 2.2, 2.4, 3.1 y 3.2)

Después de obtener un patrón constante y específico, se procedió a comprobar la capacidad de esta técnica para detectar daño en el ADN tras

exposiciones agudas y crónicas. En este proceso hubo que optimizar las concentraciones de ADN molde y cebador mas adecuadas para incrementar la sensibilidad del ensayo y evitar la presencia de falsos positivos.

Se utilizaron dos compuestos genotóxicos de referencia, la Mitomicina C (MMC) como mutágeno directo, (Anexo 2.2 y 2.4) y el Benzo(a)Pireno (BaP) como mutágeno indirecto (Anexos 3.1 y 3.2).

MMC es un mutágeno de acción directa, ampliamente conocido por su acción mutagénica y clastogénica. En función de la dosis y del periodo de exposición, éste compuesto provoca, tanto *"in vivo"* como *"in vitro"*, sustitución de bases, incremento en el intercambio de cromátidas hermanas, aberraciones cromosómicas y formación de micronúcleos. (IARC)

Los estudios de exposiciones agudas realizadas con MMC, (1 y 0,5 µg/ml durante 4 y 4, 6 y 8 h respectivamente) nos condujeron a ajustar tanto las concentraciones de ADN molde, (de 15 a 5 ng / 25 µl) como las de cebador (de 5 a 4 pM). Las condiciones finalmente fijadas resultaron óptimas en la detección de mutaciones, incluso para primers que generan un número muy diferente de productos finales (de una a seis bandas).

El BaP, se seleccionó como compuesto de referencia, en estudios agudos y en exposiciones prolongadas. La elección de dicho compuesto, además de por su relevancia ambiental, se basó en que es un mutágeno de acción indirecta, es decir, los productos activos son sus metabolitos.

El BaP pertenece al grupo de los hidrocarburos aromáticos policíclicos (PAHs) que se producen a partir de la ignición de combustibles fósiles. Está clasificado por la EPA como polucionante prioritario debido a su persistencia, carcinogenicidad y alta capacidad de acumulación a lo largo de la cadena trófica (Kanaly et al., 2001). Este compuesto, después de ser metabolizado se transforma y da lugar a formas altamente reactivas, capaces de formar enlaces covalentes con el ADN. En este tipo de estudios, donde los productos activos son los metabolitos, la línea celular RTG-2 presenta la ventaja de retener cierta capacidad metabólica, lo que le permite transformar el BaP en sus intermediarios activos (Thornton et al.,1982).

El BaP se utilizó para establecer la sensibilidad de la técnica en las condiciones anteriormente fijadas, comparándola con la inducción de micronúcleos, utilizando para ello la misma línea celular, y las mismas concentraciones y periodos de exposición. Los resultados, además de confirmar que sus efectos son dependientes del tiempo y de la dosis, mostraron que la técnica de RAPD presentaba una mayor sensibilidad. La concentración mínima de compuesto genotóxico necesario para la inducción de micronúcleos, era prácticamente el doble de la requerida para detectar alteraciones en el patrón de bandas previamente establecido. No obstante, la sensibilidad estaba en función del primer utilizado.

Las variaciones observadas en el patrón de bandas del ADN de las células expuestas, tanto a MMC como a BaP, respecto a células control,

consistieron en diferencias en la intensidad y / o en la ausencia y / o presencia de nuevas bandas.

La valoración de estas diferencias requirió el desarrollo de una metodología de análisis precisa y objetiva, para minimizar la variabilidad del método y facilitar la interpretación de resultados.

3.2.3. Sistema de Análisis para la Detección de Alteraciones en el Patrón de Bandas. (Anexo 2.2)

Como práctica habitual, y para evitar errores a lo largo del proceso, en cada gel se corrieron conjuntamente controles y expuestos (cada uno de ellos al menos por triplicado), un patrón de pesos moleculares y un blanco consistente en todos los reactivos excepto ADN molde. Además, para cada ensayo, los geles se realizan, al menos, por duplicado y en diferentes días. El análisis de las diferencias entre controles y expuestos se objetivó mediante un proceso de captación de imagen computerizado.

En el análisis del perfil fluorimétrico de cada gel, se estudiaron las diferencias cualitativas y cuantitativas. Las primeras, desaparición de bandas o la aparición de otras nuevas, se expresaron como porcentajes respecto al número de repeticiones realizadas.

Los análisis cuantitativos se llevaron a cabo mediante un software específico, considerando inicialmente tres parámetros: altura, volumen y

porcentaje de amplificación de cada una de las bandas. Las diferencias entre controles y expuestos de cada uno de estos parámetros, se establecieron mediante el análisis de la *t*-Student, confirmando los resultados mediante el "test" de *U*-Mann-Whitney. Todos los parámetros analizados mostraron diferencias estadísticamente significativas ($p<0,05$), pero el nivel de significación mas alto se obtuvo cuando se consideró el porcentaje de amplificación ($p<0,0001$). Además, y aunque los tres parámetros están relacionados, el porcentaje de amplificación resulta ser el mas objetivo, puesto que se expresa en términos relativos y facilita las comparaciones entre los perfiles de amplificación, aunque se hayan obtenido en diferentes días.

A pesar de ello, en la mayoría de los casos, los valores individualizados de cada una de las bandas presentaban un alto coeficiente de variación, por lo que se realizaron también los análisis agrupando las bandas en alto (>800 pb), medio (entre 600 y 800 pb) y bajo peso molecular (<600 pb). En estas condiciones, se observa en los perfiles de las células expuestas un decrecimiento en la amplificación de las bandas de alto peso molecular, en favor de las de bajo peso molecular. Por tanto, la agrupación de bandas en los análisis cuantitativos, disminuye la variabilidad de los datos, y muestra mas claramente las diferencias entre controles y expuestos.

La sensibilidad del ensayo se incrementó cuando se realizaron exposiciones a largo plazo. Las exposiciones crónicas ejercen una presión constante sobre las poblaciones piscícolas, que se manifiesta en muchos casos a lo largo de diferentes generaciones. Los sistemas *"in vitro"* pueden ser de

gran ayuda en los estudios en donde las observaciones de los efectos sobre la dotación genética deben realizarse tras varias generaciones.

Se expusieron células RTG-2 a una baja concentración de BaP (0,05 µg/ml) y tras 3, 15 y 30 días de exposición, se extrajo y analizó el ADN. Los resultados, al igual que los obtenidos en la exposición aguda realizada con este mismo compuesto, presentaron una relación tiempo y dosis dependiente, confirmándose también la sensibilidad selectiva de los primers utilizados. Particularmente interesante fue la observación de la aparición de nuevas bandas altamente repetitivas, tanto en el 100% de las amplificaciones como en la totalidad de los extractos analizados, tras el mayor periodo de exposición.

3.3. CAPACIDAD PARA PREDECIR EL DAÑO "IN VIVO".

(Anexos 2.3 y 4.1)

Los sistemas *"in vitro"* presentan grandes ventajas científicas técnicas, económicas y éticas, pero deben ser capaces de predecir los efectos que se observarían en la especie *"in vivo"*. Por lo tanto, la capacidad de extrapolación de los resultados es requisito fundamental, por razones obvias, para su aplicación.

En el caso concreto de la detección de alteraciones en el ADN mediante la técnica de RAPD, la capacidad predictiva del ensayo vendría determinada por:

1. **La Similitud Genética entre los Sistemas *"in vivo"* e *"in vitro,"*** que se manifiesta en el grado de semejanza de sus respectivos patrones de bandas y

2. **La Similitud de los Efectos *"in vivo"* e *"in vitro"*** producidos por un mismo agente tóxico.

3.3.1. Similitud Genética entre los Sistemas *"in vivo"* e *"in vitro"*. (Anexo 2.3)

Se comparó el patrón de bandas de la línea RTG-2 con el de los individuos de trucha arcoiris, especie de la que procede. Para ello, se utilizaron 33 individuos procedentes de 3 piscifactorías distintas. El ADN se extrajo a partir de células de sangre periférica, y las amplificaciones se realizaron mediante los cebadores inicialmente seleccionados para la caracterización de la línea celular RTG-2. Para comparar el patrón de bandas de cada uno de los individuos con la línea celular, se amplificaron los resultados conjuntamente y por duplicado en un mismo gel.

Finalizado este proceso, se constató que los extractos genómicos de todos los individuos generaban un total de 73 fragmentos frente a los 56 obtenidos en la línea celular. Este menor número de bandas está relacionado con el carácter homocigótico de la línea celular. El análisis comparativo de los patrones mostró una gran concordancia, ya que casi un 75% de las bandas se encontraron en ambos sistemas. Las restantes se debieron a la presencia de

polimorfismos.

Las amplificaciones de todos los cebadores mostraron *"in vivo"* bandas polimórficas, consecuencia de su diversidad genética, pero su número estuvo en función del cebador utilizado. La similitud observada en los patrones de bandas es una consecuencia de la homogeneidad que presentan ambos sistemas. Así, el *índice de similaridad inter-poblacional "in vivo" / "in vitro"* obtenido (0,931) está muy próximo al valor máximo 1.

Igualmente, en el dendrograma de distancias genéticas construido entre todos los individuos analizados y la línea RTG-2, se observaron dos brazos indistintamente formados por individuos pertenecientes a todas las poblaciones y la línea celular RTG-2. Como conclusión de este trabajo y para estudios posteriores, se seleccionaron de entre los primers inicialmente ensayados, aquellos cuyo patrón de bandas presentaba una mayor concordancia *"in vivo"* e *"in vitro"*.

3.3.2. Similitud de Efectos *"in vivo"* e *"in vitro"*. (Anexo 4.1)

Una vez constatada la similitud genética de ambos sistemas, se realizó un ensayo *"in vivo"* con el mismo agente (BaP), que permitiera comprobar si los efectos observados *"in vitro"* se confirmaban *"in vivo"*.

Se realizó una exposición subletal, mediante una inyección intraperitoneal, y se valoraron sus efectos a diferentes tiempos (1 - 4 meses),

comparando el patrón de bandas obtenido a partir del ADN de células de sangre periférica.

Los análisis cualitativos y cuantitativos confirmaron los resultados *"in vitro",* mostrando la aparición de nuevas bandas y alteraciones en su intensidad. No obstante, la presencia de bandas polimórficas dificultó el análisis entre los grupos de individuos tratados y no tratados, de manera que las comparaciones solamente se pudieron realizar en un mismo individuo antes y después del tratamiento. Esta es la mayor limitación que presenta la técnica de RAPD en los estudios *"in vivo"*. El sistema de agrupación de bandas se mostró especialmente adecuado para evidenciar los efectos del contaminante. No obstante, y debido al pequeño número de individuos analizados en este estudio, no es posible extraer conclusiones determinantes, ya que la propia susceptibilidad individual requiere el análisis de muestras de gran tamaño. De cualquier manera los resultados obtenidos son, sin duda, la manifestación de las alteraciones producidas por este compuesto en el material genético, tanto *"in vivo"* como *"in vitro"*, demostrando claramente la capacidad de la línea celular RTG-2, tanto para metabolizar este conocido promutágeno, como para predecir los efectos genotóxicos que se observarían en los individuos expuestos.

Con este trabajo, sólo hemos pretendido apuntar la capacidad metodológica del sistema desarrollado y, es evidente que para poder llegar a conclusiones más fiables que permitan transferir dichos resultados *"in vitro"* a los sistemas *"in vivo",* son necesarios estudios más completos y ambiciosos

que exceden de los objetivos de este trabajo.

4. DISCUSIÓN

Uno de los objetivos de la ecotoxicología, es proporcionar metodologías sensibles y realistas que permitan acometer las valoraciones de riesgo con mayor grado de seguridad. Actualmente, en los foros de consenso internacional sobre seguridad de sustancias químicas para el medio acuático (Convenio OSPAR, Convenio de Londres y de Barcelona) , se está planteando la política de eliminación de la contaminación en su origen, recomendando la adopción del concepto de sustitución de las sustancias químicas más peligrosas, o bien reduciendo sus niveles de emisión a cero. Esta política, que acarreará una gran controversia en particular por parte de la industria, se debe en gran medida a la ausencia de metodologías prácticas capaces de predecir efectos a largo plazo sobre las poblaciones expuestas. El desarrollo de nuevos métodos, que contribuyan a la monitorización de los efectos que producen los compuestos químicos de relevancia medioambiental sobre los organismos y poblaciones expuestas, es hoy en día, por tanto, una prioridad en ecotoxicología.

Las técnicas moleculares que permiten la obtención de la huella genética o patrón de bandas específico del sistema biológico utilizado, pueden aportar nuevas herramientas en la detección de efectos genotóxicos sobre los individuos y poblaciones expuestas.

La aparición a principios de los noventa de este tipo de técnicas, que no requerían el conocimiento de la secuencia de ADN, supuso una auténtica revolución en el campo de la ecotoxicología genética, pues permiten obtener

huellas genéticas especie–específicas, población–específicas e incluso individuo–específicas. Esta es una de las razones por las cuales la literatura científica que se puede encontrar sobre el tema, sea prácticamente coincidente en el tiempo y se deba en su mayoría a los trabajos de tres o cuatro equipos de investigación en todo el mundo que hemos trabajado simultáneamente.

La obtención de un patrón de bandas especifico de un determinado ADN permite detectar cambios en dicha molécula tras ser expuesta a diferentes factores de estrés, como son los contaminantes físicos o químicos (Kubota et al., 1992,1995). Con estas técnicas, la huella genética se obtiene mediante una PCR que utiliza primers cortos y de secuencia arbitraria. Se han desarrollado tres protocolos diferentes, de los cuales el de AP-PCR y particularmente el de RAPD han sido los que se han aplicado de forma mayoritaria.

Los primeros trabajos aplicaron la técnica de AP-PCR. Su protocolo utiliza altas temperaturas de hibridación, permitiendo aumentar la especificidad cebador-molde y obtener un número reducido de fragmentos finales. Por el contrario, la técnica de RAPD, debido a las bajas temperaturas que utiliza (34-39º C), permite multiplicar los lugares de unión cebador–ADN molde y por tanto aumentar el número de bandas o productos finales. Sin embargo, la falta de especificidad de muchas de estas uniones, hace que ligeras variaciones en algunos de los parámetros que intervienen en la reacción, alteren la intensidad e incluso el número de los productos amplificados. Todo ello ha contribuido a cuestionar la reproducibilidad de sus resultados y en definitiva, la idoneidad de esta técnica (Abed et al., 1995; Bassam et al., 1992; Ellsmorth et al., 1993;

Meunir and Grimont 1993; Park and Kohel, 1994; Penner et al., 1993; Regli et al., 1995).

Nuestra hipótesis inicial partió de la base de que si los diferentes parámetros que intervienen en la reacción eran minuciosamente estandarizados, los resultados llegarían a ser altamente repetitivos y por tanto la técnica podría ser aplicada satisfactoriamente en ecotoxicología. Ello ha supuesto que el trabajo realizado en esta tesis haya sido eminentemente metodológico.

Uno de los factores a controlar es la integridad del ADN utilizado como molde. Durante la fase de hibridación, el cebador busca o explora a lo largo de todo el genoma, secuencias complementarias a las que unirse. Sólo si existen lugares de unión en ambas cadenas del ADN y a distancias adecuadas, el fragmento comprendido entre ambos será amplificado. Si el ADN ha sufrido roturas durante el proceso de extracción o conservación, algunos de los lugares de unión pueden verse dañados y por tanto, afectar a la reproducibilidad de los resultados finales. De la gran cantidad de métodos de extracción, incluyendo “kits” comerciales, de que se dispone en la actualidad, sólo pueden utilizarse aquellos que no rompan la molécula de ADN (Black, 1993; Cheng et al., 1995). Según nuestros resultados, la extracción con fenol cloroformo proporciona un ADN de alta eficiencia que puede ser almacenado durante varios meses a 4º C sin alterarse, lo que le convierte en un método óptimo.

Además, en la reacción de amplificación propiamente dicha, intervienen un gran número de variables que cada equipo de investigación ha protocolizado a su elección. Algunas de ellas están condicionadas por el tipo de polimerasa y el ciclo de temperaturas elegido (Roehrdanz y col., 1993; Penner y col., 1993; Meunier and Grimont 1993). La Taq polimerasa fue empleada por Williams y colaboradores en 1990 en el desarrollo del método, pero en la actualidad el fragmento Stoffel de la Taq polimerasa esta siendo preferentemente utilizado, pues a bajas temperaturas de hibridación proporciona resultados más reproducibles y presenta una actividad óptima dentro de un rango más amplio de concentraciones de iones magnesio (Nicol et al., 1998; Schiliro et al., 2001; Aljanabi et al., 1999; Lawyer et al., 1993).

Algo semejante ha sucedido con el ciclo de temperaturas. La unión cebador–ADN molde es directamente dependiente de la temperatura de hibridación y pequeñas variaciones en ella pueden modificar los lugares de unión y, por tanto, los productos finalmente amplificados. Penner y col., (1993) en un estudio comparativo, comprobaron que la diferencia en los resultados finales entre distintos laboratorios, era debida a las diferencias de temperaturas conseguidas por los termocicladores en el interior de los tubos de amplificación, a pesar de estar igualmente programados. Williams, y col., (1990) al desarrollar el método sugirieron la imposibilidad de utilizar temperaturas superiores a 40º cuando se trabajaba con primers cortos de 10 pares de bases. Posteriormente, sin embargo, se han desarrollado protocolos para la detección de mutaciones en el intervalo de temperaturas que va desde 35ºC (Conte et al., 1998) a 50ºC (Atienzar et al., 2000b). En nuestro caso la temperatura óptima se ha fijado en

36º C.

La concentración de ADN molde y primer son factores que afectan en la obtención de un patrón de bandas constante. Aunque existen diferentes opiniones sobre su influencia (Van Belkum 1994; Welsk and McClelland 1990, Muralidharan and Wekeland 1993; Davin-Regli et al., 1995), en nuestros estudios han resultado ser de gran importancia para la obtención de resultados repetitivos y para eliminar la presencia de bandas erráticas. No obstante, hemos podido comprobar que cada cebador, en función del número de bandas que amplifica, presenta concentraciones óptimas de trabajo, lo que posiblemente sea la causa de las diferentes opiniones al respecto. Además, la concentración de ADN molde fue decisiva para la detección de diferencias en el ADN de células control y expuestas a compuestos genotóxicos, y su optimización ha permitido aumentar la sensibilidad, eliminando la presencia falsos positivos. El ADN de las células expuestas, en función de las concentraciones y tiempos de exposición utilizados, permanece intacto en un cierto número de copias. Cuando se utilizan concentraciones de ADN altas (20 ng / 25 µl), el número de copias inalteradas es suficiente para que el primer (4 pM) pueda encontrar secuencias donde hibridar y dar lugar, finalmente, a patrones de bandas no alterados. Sin embargo, cuando la concentración de ADN es menor, (5 ng / 25 ul) y a pesar de que en términos relativos la proporción de copias intactas sea la misma, el numero absoluto es mas bajo e insuficiente, incrementando así la probabilidad de mostrar secuencias alteradas.

El ajuste de la concentración de ADN a cantidades tan bajas, requiere una gran precisión para la cuantificación de los extractos, en este sentido, una lectura adicional a 320 nm permite eliminar las interferencias debidas a otros componentes presentes en el medio, evitando la sobrevaloración de la medida.

Además de las variables consideradas, la obtención de un patrón de bandas específico y adecuado para la detección de genotoxicidad requiere una meticulosa selección de los primers. Del total de los mismos y sus combinaciones binarias inicialmente probadas, sólo se seleccionaron 8, siguiendo como criterio que las bandas amplificadas sean constantes y bien definidas, y que se encuentren dentro de un amplio rango de pesos moleculares. La necesidad de una buena reproducibilidad del patrón resulta obvia, y la obtención de bandas de diferentes pesos moleculares, incrementa la sensibilidad en la detección de daño al ADN. (Atienzar, et al., 2001)

Por lo que respecta a la aplicación de la técnica de RAPD para el estudio de alteraciones genéticas *"in vivo"*, existen ciertas complicaciones. Los individuos pertenecientes a una misma especie, presentan diferencias individuales que se manifiestan como bandas polimórficas. Este hecho, que es de gran interés para estudios de diversidad, es un grave inconveniente cuando se aplica a la detección de genotoxicidad, pues la presencia o ausencia de bandas entre los individuos expuestos y no expuestos, puede ser debida tanto a la presencia de polimorfismos propios de la especie, como a la acción de sustancias genotóxicas, lo que implica, para evitar incertidumbre, la utilización de muestras de gran tamaño, encareciendo y complicando el estudio

(Theodorakis, et al., 1997; 1998; Krane et al., 1998; Bickham et al., 2000). La utilización de individuos genéticamente homogéneos, tales como clones de *Daphnia magna* (Atienzar et al., 1999; 2001) o células pertenecientes a una misma línea celular, permite detectar fácilmente tales cambios, tras comparar los patrones de bandas de individuos controles y expuestos.

La utilización de cultivos celulares en la detección de efectos genotóxicos simplifica muchos de los problemas asociados al uso de sistemas *"in vivo",* y pueden resultar de gran utilidad en los procedimientos de predicción / valoración de riesgo, siempre y cuando se haya constatado el grado de similitud entre ambos sistemas. Hay que tener en cuenta que las líneas celulares establecidas pueden poseer características genéticas diferentes de las células originales y, además, muchas especies de peces presentan una gran variabilidad en su dotación genética. Por ejemplo, en trucha arcoiris se han descrito individuos con dotaciones diplóides, tetraplóides e incluso aneuplóides. (Al-Sabty, 1991). Por ello es imprescindible comparar ambos patrones de bandas (*"in vivo"*/*"in vitro"*) y establecer su grado de similaridad, mediante la detección de bandas comunes y / o exclusivas de cada uno de los sistemas, con objeto de acotar mejor la extrapolación de resultados.

La comparación de los patrones de bandas de diferentes individuos de trucha arcoiris y de la línea celular RTG-2 utilizando los 8 primers seleccionados, reveló una gran concordancia, pues el 73% de las bandas que componen el patrón de la línea celular RTG-2 están presentes en todos los individuos de trucha arcoiris estudiados. La línea celular presenta un numero

menor de bandas que el sistema *"in vivo",* como consecuencia de su carácter homocigótico (Theodorakis, et al., 1997). Debido a que el numero de loci que pueden ser analizados por esta técnica es teóricamente ilimitado (Lynch and Milligan, 1994), aproximadamente el 50% de las bandas obtenidas *"in vivo "* resultaron ser polimórficas, mostrando la variabilidad genética de las poblaciones estudiadas.

A pesar de que la técnica de RAPD es relativamente nueva y hay una información limitada de valores de referencia, el índice de similaridad interpoblacional *"in vivo"/"in vitro*" obtenido (0,931) es considerablemente mayor al mostrado por otros autores entre poblaciones piscícolas dentro de una misma especie. (Bardakci et al., 1994; Bielawski and Pumo, 1997).

Teniendo en cuenta la gran similitud genética que presentan ambos sistemas, cabría esperar efectos genotóxicos similares cuando individuos de trucha arcoiris y células RTG-2 estén expuestos a un mismo contaminante. Esta hipótesis quedó demostrada al detectar alteraciones en el patrón de bandas de células RTG-2 y de células de sangre periférica de individuos de trucha arcoiris expuestos a BaP. Sin embargo, en los estudios *"in vivo",* la propia susceptibilidad individual y la presencia de polimorfismos hace que los resultados no sean homogéneos, obligándonos a realizar análisis horizontales para detectar en un mismo individuo, las variaciones que sufre su patrón de bandas antes y después de la exposición. Esta aproximación experimental tiene cabida en los estudios de laboratorio cuando el tamaño de la especie lo permite, pero desde luego no es posible cuando se pretende realizar un estudio

de monitorización de campo, a menos que se incremente notablemente el tamaño de la muestra.

En los estudios de campo con poblaciones naturales se puede, tras estudiar un elevado numero de individuos, determinar la frecuencia de aparición de las distintas bandas dentro de una población expuesta y compararlo con poblaciones no expuestas o controles. Aunque se han abordado algunos estudios de este tipo, (Theodorakis et al., 1997; 1998; Krane et al., 1998) la dificultad que presenta el disponer de poblaciones control, y los problemas inherentes a los propios estudios de campo (sexo, tamaño, edad de los individuos capturados, etc.), limita en gran medida su realización.

Estos problemas pueden ser minimizados utilizando sistemas biológicos genéticamente homogéneos, donde una población celular puede ser expuesta a la acción de un determinado genotóxico durante varias generaciones, debido a su, relativamente corto, ciclo celular. Concretamente en los estudios que hemos realizado con mitomicina C y benzo(a)pireno, el patrón de bandas obtenido para la línea celular RTG-2, ha mostrado ser sensible a tales cambios, detectándose tanto variaciones cualitativas (alteraciones en la frecuencia de aparición de alguna de las bandas y / o presencia de otras nuevas), como cuantitativas (variaciones en su intensidad de amplificación). Estos cambios cualitativos, detectados entre los patrones controles y expuestos, han sido confirmados por otros autores, tanto utilizando el mismo compuesto - BaP-, como en exposiciones a otro tipo de contaminantes - luz ultravioleta y metales pesados - en una amplia variedad de especies: gusanos (*Platynereis dumerilii*),

moluscos (*Mytilus edulis*), microcrustáceos (*Daphnia. magna*), plantas acuáticas (*Arabidopsis thaliana*), algas (*Palmaria palmata*) y –bacterias (*Escherichia coli* y *Renibacterium salmoninarum*) (Saava 1996; Atienzar et al., 1999; 2001; 2000a; Conte et al., 1998).

La desaparición y/o aparición de nuevas bandas, revelan cambios en la estructura del ADN debido a la presencia de mutaciones puntuales que alteren o generen nuevos lugares de unión con el primer, a delecciones que provoquen la pérdida de algunos lugares de unión y acerquen lugares de hibridación ya existentes, o como han sugerido diversos autores, como consecuencia de la recombinación de homólogos, yuxtaponiéndose dos secuencias complementarias a la secuencia del primer. (Kubota et al., 1992; 1995; Peinado 1992; Atienzar 1999; 2001, Saava, 2000; Conte et al 1998).

La aparición de nuevas bandas como consecuencia de mutaciones requiere que éstas se produzcan en un mismo locus en un número suficientemente alto de células, al menos en el 10%, para conseguir que un nuevo producto de PCR sea visible en geles de agarosa (Atienzar et al., 1999). La obtención de una nueva banda con un alto porcentaje de aparición, podría sugerir la interacción del tóxico con el ADN en lugares específicos de la molécula, mostrando de esta forma la existencia de puntos calientes o secuencias específicamente sensibles a la acción de un determinado tipo de agente inductor.

En las exposiciones a corto y largo plazo que hemos realizado con BaP,

se muestra la aparición, en los patrones de las células expuestas, de dos nuevas bandas (D-8_{936} y AA-82_{620}) de pesos moleculares iguales en ambos ensayos. Esto muestra la capacidad de la técnica para detectar la presencia de puntos calientes en el genoma a la acción del tóxico, como ya observamos en exposiciones agudas a MMC y como también ha sido sugerido por otros autores (Atienzar et al., 2001; Saava 1998).

En las exposiciones prolongadas (15-30 días) a bajas concentraciones de BaP (0,05 µg/ml), la manifestación de estos efectos resulta más evidente. A los 15 días de exposición, se observó la presencia de cuatro nuevas bandas aunque con diferentes porcentajes de aparición. Esta variabilidad fue debida, tanto a que durante este periodo de exposición sólo afecta a parte de población celular, como a la propia dinámica de la reacción, donde los productos finales son el resultado de una multiplicación exponencial de los fragmentos mas abundantes, lo que hace que en ocasiones esas bandas no sean reproducibles al 100% (Davin-Regin 1995). Al aumentar el periodo de exposición a 30 días, solo persistieron dos de ellas (AA-82_{620} y AA-89_{484}) pero con un porcentaje de aparición del 100%, sugiriendo que dicha alteración se encuentra en un numero elevado de células; es decir, se produce un aumento en el índice de mutación, y lo que es más, sugiere el carácter hereditario de dicha alteración genética.

Las implicaciones biológicas que pueden acarrear estos cambios están en función de la secuencia afectada. Pueden darse en genes recesivos, trasmitiéndose a las células hijas y manifestándose ante un cambio en las condiciones ambientales, o bien, pueden producirse en secuencias que

codifican proteínas vitales para la supervivencia de las células, dando lugar a procesos de letalidad a corto o medio plazo.

Por lo que nosotros hemos podido constatar, los equipos de investigación que han utilizado y optimizado la técnica de RAPD para la detección de mutaciones, se han centrado en el análisis cualitativo o semicuantitativo de los datos (Conte et al., 1998; Atienzar et al., 1999; 2001; Theodorakis et al., 1997). Nuestro desarrollo metodológico ha ido encaminado a dotar de mayor precisión a la técnica, por lo que, cuando los resultados se analizan mediante programas de ordenador específicos, además de eliminar la subjetividad propia del observador, el análisis puede realizarse de forma cuantitativa utilizando la altura, el volumen y el porcentaje de amplificación de cada una de las bandas. De todos ellos el más adecuado, en nuestra opinión, es el porcentaje de amplificación, pues proporciona valores relativos al total amplificado, permitiendo una mejor comparación entre los diferentes geles y al mismo tiempo, una menor dispersión. Además, cuando las bandas se agrupan según sus pesos moleculares, las diferencias se manifiestan más claramente, mostrando en los individuos expuestos una pérdida de amplificación de las bandas de alto peso molecular a favor de las de bajo peso molecular.

Aunque la causa de las diferencias de intensidad se deba a un conjunto de eventos, la más significativa probablemente sea la pérdida de amplificación, bien por la formación de aductos que bloqueen la polimerización del ADN (Nelson et al., 1996), o por la presencia de roturas en dichos lugares (Atienzar et al., 1999). En ambos casos los fragmentos de mayor peso molecular tienen

una mayor probabilidad de sufrir tales eventos (Atienzar et al., 1999), y en consecuencia, la eficiencia de la amplificación se desplaza hacia los fragmentos de menor peso molecular. En el caso de las exposiciones agudas a MMC, observamos variaciones en la razón *alto:bajo* peso molecular de 5:2 en los controles, a 1:1 en los expuestos. Aunque desde luego es imprescindible realizar estudios más amplios tanto a lo que se refiere al número y tipo de compuestos, como al número de individuos; nos atrevemos a sugerir que tal vez la utilización de esta sencilla relación matemática pueda servir como parámetro o biomarcador de genotoxicidad.

En resumen, las modificaciones en el patrón de bandas pueden estar originadas por un amplio rango de alteraciones en el ADN, desde cambios de bases (mutaciones puntuales) a complejos reagrupamientos de cromosomas (White et al., 1990; Peinado et al., 1992; Welsh et al., 1991 Kubota et al., 1995; Atienzar et al., 1999; 2000; 2001). La capacidad de la técnica para detectar un amplio espectro de alteraciones estructurales en el ADN la hace especialmente interesante para los estudios ecotoxicológicos, pues incrementa de 2 a 4 veces su sensibilidad, concretamente en el caso del BaP, frente a otros ensayos clásicos de genotoxicidad, como el incremento de micronúcleos (Kolpoth et al., 1999; Sánchez et al., 2000) o aberraciones cromosómicas (Kocan et al., 1982 y 1985), utilizando la misma línea celular, o bien a escala poblacional detectando inestabilidad genética frente a otros parámetros poblacionales como crecimiento, supervivencia, fecundidad, etc. (Atienzar et al., 1999; 2000; 2001), con este mismo genotóxico.

Por otro lado no todos los primers presentaron la misma capacidad para detectar alteraciones, ya que de los cuatro utilizados, dos se mostraron más sensibles a la acción del tóxico (AA-82 y AA-89). Fueron estos mismos, los que manifestaron los mayores porcentajes de polimorfismos (>50%) en los individuos de trucha arcoiris analizados, sugiriendo la posibilidad de que las secuencias que amplifican presenten una alta susceptibilidad a ser alteradas por agentes externos.

En definitiva, la técnica de RAPD se muestra como una valiosa herramienta para la detección de efectos sobre el ADN. La rapidez, versatilidad y sensibilidad para detectar un amplio rango de alteraciones son sus principales ventajas. Además, su aplicación conjunta con sistemas *"in vitro",* cuya homogeneidad genética elimina la presencia de polimorfismos, permite detectar alteraciones transgeneracionales en exposiciones prolongadas a genotóxicos medioambientalmente relevantes por su persistencia y ubicuidad contribuyendo a dotar de mayor grado de seguridad a los procedimientos de valoración de riesgo.

5. CONCLUSIONES.

1. La extracción del ADN molde con fenol cloroformo, proporciona una alta eficiencia de amplificación y permite que los extractos genómicos puedan utilizarse, almacenados a 4º C, por periodos de hasta nueve meses.

2. Para evitar la sobrevaloración de la concentración y pureza del ADN presente en los extractos, es necesario sustraer el fondo inespecífico, realizando una medida espectrométrica adicional a 320 nm.

3. Para eliminar la presencia de falsos positivos, la integridad del ADN extraído en cada uno de los extractos debe ser comprobada mediante geles de agarosa antes de iniciar la amplificación.

4. El protocolo que proporciona mayor grado de reproducibilidad consiste en: 4 mM de Mg, 0,2 mM de cada dNTP, 2 U del fragmento Stofel, 5 pM de primer, 15 ng de ADN en un volumen finadle 25 μl; una temperatura de hibridación de 36º C durante 75 seg y un numero de 50 ciclos. La visualización de los fragmentos amplificados se realiza mediante electroforesis a 115 v durante 4 h en geles de agarosa al 2,1%.

5. El patrón de bandas o huella genética de la línea celular RTG-2 quedó

establecida con 8 cebadores que generaban un total de 56 bandas, con una reproducibilidad superior al 80% en todas las amplificaciones y dentro de un rango de pesos moleculares de entre 2000 y 300 pares de bases.

6. Este patrón de bandas es sensible a la detección de alteraciones en el ADN de células expuestas, tanto a genotóxicos de acción directa, mitomicina C, como indirecta, benzo(a)pireno; esta sensibilidad varía en función del cebador utilizado.

7. Aunque el patrón de bandas obtenido es constante dentro de un amplio rango de concentraciones de ADN y cebador, la sensibilidad en la detección de daño al ADN fue óptima cuando éstas se ajustaron a 5 ng de ADN y 4 pM de cebador.

8. El daño al ADN se detecta como variaciones en el patrón de bandas de las células expuestas, respecto al de las células control. El sistema desarrollado permite evidenciar alteraciones cualitativas (aparición de nuevas bandas y / o perdida de la amplificación de alguna de ellas) y / o cuantitativas (variaciones en la intensidad de amplificación)

9. De los parámetros considerados para el análisis cuantitativo, altura volumen y porcentaje de amplificación de las bandas, se selecciono este último ya que presenta menor dispersión de datos, y además permite el análisis de resultados obtenidos en diferentes días.

10. La agrupación de bandas en los análisis cuantitativos en alto, medio y bajo peso molecular, disminuye la variabilidad de los datos, y muestra mas claramente las diferencias entre genomas controles y tratados. Estas diferencias se manifestaron en los genomas expuestos como un decrecimiento en las bandas de alto peso molecular a favor de las de bajo peso molecular.

11. El sistema desarrollado resultó ser de 2 a 4 veces más sensible que otros ensayos clásicos de genotoxicidad, utilizando la misma línea celular, el mismo compuesto tóxico y periodos de exposición.

12. El patrón de células sanguíneas de trucha arcoiris, presenta un número mas elevado de bandas que el obtenido para la línea RTG-2 (73 frente a 56). El 50% de las bandas fueron polimórficas. Los primers AA-89 y AA-82 mostraron los porcentajes de polimorfismos mas elevados.

13. El análisis comparativo de los patrones *“in vivo” / ”in vitro”* mostró una gran semejanza. Alrededor de un 75% de las bandas se encontraron en ambos sistemas. Los primers AA-89, AA-82, D-8 y C-96 se seleccionaron por su mayor concordancia para posteriores estudios.

14. La línea celular RTG-2 y la especie de la que procede presentan una gran homogeneidad genética, con un alto *índice de similaridad Inter.-*

poblacional "in vivo" e "in vitro" (0.931). Igualmente el dendrograma de distancias genéticas construido entre todos los individuos analizados y la línea RTG-2, no muestra diferencias entre los individuos trucha arcoiris y la línea celular RTG-2.

15. En los estudios in vivo tras la exposición intraperitoneal de 69 μg/g p.c. a Benzo(a)pireno se detectaron alteraciones cualitativas, presencia de una nueva banda, y cuantitativas, diferencias en los porcentajes de amplificación, en todos los individuos de trucha arcoiris. Estas alteraciones confirmaron las observadas en los estudios *"in vitro" .*

16. La presencia de nuevas bandas, como consecuencia de alteraciones en lugares específicos del genoma observadas tanto *"in vivo"* como *"in vitro",*, hace suponer la presencia de secuencias más sensibles a la acción del toxico. La capacidad de la metodología desarrollada para evidenciar estas secuencias más sensibles en la molécula de ADN, resulta de una gran utilidad para el estudio de los mecanismos de acción de genotóxicos ambientales.

17. La presencia de bandas polimórficas, obliga a realizar estudios horizontales en cada individuo, antes y después de la exposición. Esto limita y dificulta los estudios de campo marcando como requisito imprescindible la utilización de muestras de gran tamaño.

18. El sistema desarrollado mediante la combinación de ensayos *in vitro* y

la técnica de RAPD proporciona sensibilidad, versatilidad y rapidez, lo que nos induce a recomendarlo para que sea incorporado, como una herramienta muy útil, en los procedimientos de valoración de riesgo para el medio acuático de sustancias químicas y mezclas complejas.

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II. ANEXOS

ANEXO 1

PROTOCOLO DEL ENSAYO

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1. MATERIAL

1.1. CELULAS

Se utiliza la línea celular certificada RTG-2 CCL55 de la American Type Culture Collection procedente de tejido gonadal de Oncorhynchus mykiss de morfología fibroblástica, establecida por Wolf y Quimby en 1962.

1.2. EQUIPOS

- Analizador de imagen.Gelwork 1D (UVP)
- Autoclave. Selecta.
- Baño termostatizado. Selecta
- Bombona de CO_2
- Cabina de flujo laminar vertical. Testar.
- Captador de imagen. Grab-it (UVP)
- Centrífuga. Eppendorf
- Congelador a – 40º C
- Cubetas (varios tamaños). Ecogen
- Cubetas de cuarzo. Hellma.
- Espectrofotometro. Perkin-Elmer
- -Estufa incubador a 20º C. Nuaire
- Frascos de PVC para cultivos de células de 75 cm^2 . Costar
- Fuente de Alimentación. Apelex

- Micropipetas de volumen variable (0.5 – 25, 25 – 100 / 100 – 200 / 200 – 1000 μl). Wilson.
- Microscopio invertido. Olimpus.
- Nevera a 4º C. Indesit
- Peines. Ecogen
- Pipetas estériles desechables de 1, 5, 10 ml. Corning.
- Portageles. Ecogen.
- Puntas estériles con filtro de
- Termociclador. Perkin-Elmer modelo 2400.
- Transiluminador. Espectroline.
- Tubos Eppendorf

1.3. REACTIVOS

- 2´-deoxinucleóxidos-5´-trifosfatos (dNTPs). Pharmacia.
- 8-hidroxiquinoleina. Merck.
- Acido acetico. Merck
- Ácido bórico. Serva.
- Ácido Clorhídrico. Merck
- Ácido etilen-diamino-tetra-acetico (EDTA). ICN.
- ARNasa libre de ADNasa. Bohering
- Cl_2Mg (25 mM). PERKIN- ELMER
- Cloroformo. Panreac.
- Cloruro magnesico. Perkin-Elmer.
- Cloruro potásico. Merck.

- Cloruro potásico. Merck.
- Dihidrogeno fosfato de potasio. Merck.
- Dodecil sulfato sodico (SDS). Merck
- Estreptomicina. Flow.
- Fenol. Merck
- Fragmento Stoffel, enzima polimerasa (10 U/µl). PERKIN- ELMER
- Fungizona 250 µg/ml (Amphotericina B). Flow.
- Glucosa. Flow.
- Hidrógeno fosfato de disódio. Merck.
- Hidróxido Sódico. Merck.
- L-Glutamina (200 mM). Flow.
- Mercaptoetanol. Serva.
- Penicilina. Flow.
- Solución buffer 10X (ClK 100 mM, EDTA 0.1 mM, Tris-ClH 20 mM,). PERKIN-ELMER
- Suero fetal bovino. (SFB) Flow.
- Tripsina. Flow.
- Alcohol etílico. Merck
- Alcohol isoamilico. Quimon.
- Azul de bromofenol. Serva.
- Oligonucleotidos o primers: AA-89 (5´-GGGCCTCTGAT–3´), C-95 (5´-CGGCCACTGT–3´),C-96 (5´-AGCACTGTCA-3´), D-4 (5´-CTGTAGCATC-3´), D-8 (5´-CCAAGTCGACA-3´), Trna-1 (5´-AGTCCGGTGTGCTCTA-3). Pharmacia
- Tris-hidroxi-metil-amino-metano (Tris-base). ICN.

- Verseno. Flow.
- Xilen-cianol. Bioprove.
- Agarosa.
- Agua milliQ. esteril
- Bromuro de Etídio. Bioprobe.
- Marcador de Peso Molecular (poner los dos)
- Medio Eagle con sales de Earle con aminoácidos no esenciales, penicilina (200 IU/ml), estrectomicina (100 µg/ml) y 0.85 g/l de bicarbonato sódico (EMEM). Flow.

1.4. SOLUCIONES QUÍMICAS Y MEDIOS DE CULTIVOS

1.4.1. Utilizados en el mantenimiento de la línea celular.

Medio de cultivo: (medio 1)

EMEM	500 ml
L-Glutamina (200mM)	5 ml
Fungizona	2.5 ml
Suero fetal bovino	50 ml

- Solución tampón fosfato (PBS) (Dulbecco y Vogt, 1954). Se prepara con agua milliQ y se ajusta pH 7.2

Solución 1.- solución de Tripsina-Verseno

ClNa 16 g., ClK 0.8 g., PO_4HNa_2-$12H_2O$ 0.24 g., PO_4H_2K 0.12 g., Glucosa 2.2., Rojo Fenol 0.02 g., Tripsina 2.5 g., Verseno 0.275 g.,

CO_3HNa 1.4 g., Penicilina 200.000 U. Colimicina 400.000 U. Estreptomicina 0.160 g.

Se enrasa a 1000 ml con agua milliQ. Una vez preparada se esteriliza por filtración, se hacen alícuotas y se congela hasta su uso a –20º C.

1.4.2. Utilizados en la extracción de ADN.

Solución 2.- solución buffer Tris-base 1 M.

Tris-base	48,44 g
Agua milliQ.	400 ml

Solución 3.- solución buffer Tris-ClH 1M

Tris-base	12,11 g
ClH	4.2 ml
Agua milliQ.	100 ml

Una vez preparada ajustar a pH 8 con ClH y autoclavar. Almacenar a temperatura ambiente.

Solución 4.- solución buffer Tris-ClH 0.1M.

A partir de la solución 3 efectuar una dilución 1:10 utilizando agua milliQ.

Solución 5.- solución Tris-EDTA (TE).

Tris-base	0.0302 g
EDTA	0.93 g
Agua milliQ.	Hasta 25 ml

Ajustar el pH a 8 con NaOH. Autoclavar y guardar a temperatura ambiente.

Solución 6.- solución buffer de extracción (EB).

Tris- base	0.6056 g
EDTA	0.9304 g
ClNa	1.45 g
Agua milliQ.	Hasta 50 ml

Ajustar a pH 8. Autoclavar y guardar a temperatura ambiente.

Solución 7.- solución de acetato potásico 5 M.

Ajustar el pH a 7.5 con ácido acético. Autoclavar y almacenar a –20º C en alícuotas de aproximadamente 1.5 ml. Una vez descongeladas no volver a congelar.

Solución 8.- solución de fenol estabilizado con Tris-ClH.

a) Fundir el fenol en un baño a 68º C.

b) Añadir 8-hidroxiquinoleina a una concentración final de 0.1%

c) Estabilización con solución 2, para ello:

- Añadir un volumen de solución 2 (sin ajustar pH).
- Agitar 30´ y dejar en reposo durante 10´
- Añadir unas gotas de ClH y dejar en reposo durante 10´
- Desechar la fase acuosa.

d) Repetir si es necesario el paso **c)** hasta que el pH de la fase orgánica sea aproximadamente de 7.

e) Estabilización con solución 4.

- Lo mismo que el paso **c)** pero con la solución 4.

f) Repetir el paso 5 hasta que el pH de la fase orgánica sea 7.8.

g) Añadir a la fase orgánica:

- 0.1 volumen de solución 4.
- 0,2% mercaptoetanol

Almacenar en refrigeración a 4º C en oscuridad.

Solución 9.- solución de extracción.

Solución 8.	25 ml
Cloroformo	24 ml
Alcohol isoamilico	1 ml

Mezclar los reactivos en agitador magnético y dejar en reposo toda la noche. Almacenar a 4ºC en oscuridad.

Solución 10.- solución de ARNasa.

ARNasa	20 mg
Tris-ClH, 10 mM, pH 7.5	2 ml
ClNa	0.0017 g

Una vez añadidos todos los componentes calentar a 100º C durante 15´ y dejar enfriar lentamente a temperatura ambiente. Hacer alícuotas de 0.5 ml y almacenar a –20º C.

Solución 11.- solución de SDS al 10%.

SDS	1,5 g
Agua milliQ	Hasta 15 ml

Una vez preparada, ajustar el pH a 7.2 con ClH. Es conveniente hacer alícuotas de aproximadamente 3 ml.

1.4.3. Soluciones para la reacción de RAPD.

Solución 12.- solución de dNTPs.

A partir del reactivo comercial 0.1 M. Añadir 5.5 µl de cada nucleotido (dATP, dTTP, dGTP, dCTP y llevar a 220 ml con agua milliQ. Guardar a –20º C

Solución 13.- soluciones individualizadas de cada uno de los primers.

Ajustar cada uno de los primer con agua milliQ. a una concentración final de 5 pM / µl. Realizar alícuotas de 200 µl y guardar a –20º C

1.4.4. Soluciones para electroforesis.

Solución 14.- solución de Buffer Tris-borato-EDTA (TBE) 5X.

Tris-Base	54 g.
Acido borico	27.5 g.
EDTA	3.7223 g.

Agua milliQ.	Hasta 1 L

Una vez preparado ajustar a pH 8.

Tras largos periodos de tiempo pueden aparecer precipitados en cuyo caso es conveniente desechar la solución

Solución 15: solución de Buffer Tris-borato-EDTA (TBE) 0.5X

A partir de esta solución 13 realizar una dilución 1:10 con agua milliQ.

Solución 16.- solución de buffer de carga**.**

Xilen-cianol	0.025 g.
Azul de bromofenol	0.025 g.
Glicerina	3 g.
Agua milliQ.	Hasta 10 ml.

Realizar alícuotas de 1.5 ml y conservar a 4º C.

Los colorantes migran en el campo magnético de la siguiente forma: Azul de bromofenol: 300 pares de bases. Xilen-Cianol: 3000 pares de bases.

Solución 17.- solución de bromuro de etídio.

Bromuro de Etídio	0.1 g.
Agua milliQ.	Hasta 10 ml.

Conservar a 4º C en oscuridad.

2. METODOS.

2.1. CULTIVO DE CELULAS.

La línea celular RTG-2 es una línea establecida de naturaleza fibroblastica que crece adherida al sustrato formando un tapiz en monocapa celular.

Todo el manejo, crecimiento, renovación del medio y subcultivos se realizan en condiciones de esterilidad en cabina de flujo laminar y en condiciones de esterilidad.

Se hacen crecer las células en frascos de PVC de 75 cm2 de superficie, con medio EMEM con sales de Eagle, antibióticos, glutamina, 10% de suero fetal bovino (Medio1).

Para proceder a la extracción de ADN se elimina el medio y se desorganiza el tapiz celular poniéndolo en contacto con la solución 1. De esta forma se obtiene una suspensión celular.

2.2. PROCEDIMIENTO DE EXTRACCIÓN DE ADN.

A partir de células RTG-2:

La suspensión celular obtenida, se recoge en un tubo eppendof esteril y se centrifuga a 3000 rpm, durante 10´. Retirar el sobrenadante y añadir un

volumen aproximado de 1 ml de PBS repitiendo de nuevo la centrifugación. Retirar el sobrenadante.

A partir de células sanguíneas:

Añadir 1 ml de PBS a 100 μl de sangre heparinizada, centrifugar a 1500 rpm durante 10´ Retirar el sobrenadante y repetir el proceso hasta conseguir un precipitado limpio.

En precipitado obtenido a partir de células RTG-2 o células sanguíneas se procede como sigue:

a) Añadir al precipitado un volumen de 500 μl de la solución 6.

b) Añadir 35 μl de la solución 11 y calentar en baño a 65º C durante 15´

c) Añadir 165 μl de la solución 7, agitar y dejar reposar a 4º C durante 10´.

d) Centrifugar a 13000 rpm durante 10´ a 4º C.

e) Traspasar el sobrenadante a un nuevo tubo eppendorf y desechar el precipitado.

Eliminación del RNA y digestión de proteínas.

f) Añadir 20 μg de solución 10 e incubar a 37º C durante 1 h.

g) Añadir una punta de espátula de proteinasa K, agitar e incubar en estufa a 37º C, durante una noche.

Purificación del ADN.

h) Añadir 500 µl de la solución 9, centrífugar a 13000 rpm durante 3`a 4º C.

i) Recoger la fase acuosa en un nuevo tubo eppendorf y repetir el proceso hasta que la interfase esté limpia.

j) Recoger la fase acuosa y añadir 500 µl de cloroformo y centrifugar a 13000 rpm, 3`, 4º C

Precipitación del ADN

k) Recoger la fase acuosa y añadir 500 µl de isopropanol, centrifugar a 13000 rpm durante 10'.

l) Eliminar el sobrenadante y el precipitado de ADN lavarlo dos veces. La primera con Etanol 100% y tras centrifugar a 13000 rpm 10´ se repite el procedimiento lavando con etanol al 70%.

m) Secar el precipitado y resuspenderlo en agua destilada estéril. Guardar en nevera a 4º C hasta su cuantificación.

2.3. DETERMINACIÓN DE LA CONCENTRACIÓN Y PUREZA DEL ADN PRESENTE EN EL EXTRACTO.

Ambas se determinan espectrofotometricamente de la siguiente forma:

a) Añadir en una cubeta de cuarzo, 200 µl del extracto anteriormente obtenido y realizar en el espectrofotometro tres lecturas a 260, 280, 320 nm.

b) La pureza del ADN extraído se calcula mediante la relación 260/280 una vez que a ambas lecturas se les ha sustraído el valor

obtenido a 320 nm. Se considera una pureza adecuada cuando dicha relación esta próxima a 2.

c) La concentración de ADN se obtiene mediante la lectura a 260 nm una vez sustraído el valor a 320 nm (50 mg/ml equivale a 1 unidad de absorbancia).

2.4. REACCION DE RAPD

2.4.1. Componentes de la reacción.

La reacción se realiza en tubos PCR de pared fina. El volumen final de la reacción es de 25 µl .

Añadir los siguientes reactivos en el tubos de reacción:

Reactivos	Volumen	Concentración final
• Solución buffer 10 X	2,5 µl	
- Tris-ClH		**10 mM**
- KCl		**50 mM**
• Cl_2Mg	4 µl	**4 mM**
• Solución 12 (dNTPs)	2 µl	**0.2 mM de cada dNTPs**
• Solución 13 (primer)	0.8 µl	**4 pM**
• Enzima	0.2 µl	**2 U**
• ADN molde	5 ng	**5 ng / 25 µl**
• Agua esteril milliQ	Hasta 25 µl	

Uno de los tubos se tomara como blanco añadiendo todos los reactivos excepto el ADN molde.

Tapar cuidadosamente los tubos PCR e introducirlos en el termociclador. previamente programado.

2.4.2. Ciclo de temperaturas.

El ciclo de temperatura establecido es el siguiente:

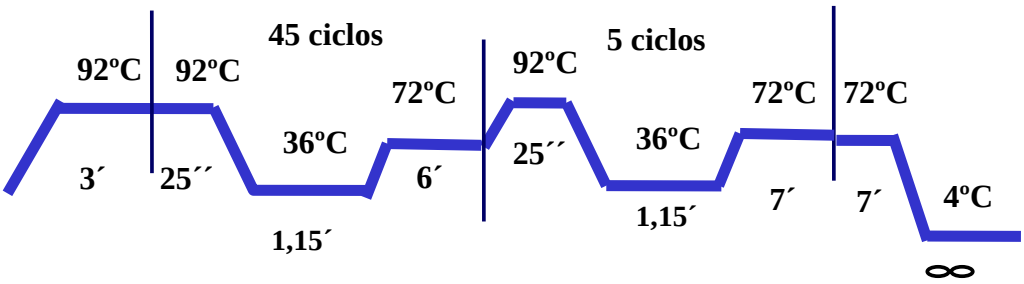
Inicialmente 92º C durante 3´ seguido de cuarenta y cinco ciclos programados de la siguiente forma:

Desnaturalización: 92º C durante 25 seg.

Hibridación: 36º C durante 1 min. 15 seg.

Elongación: 72º C durante 6 min.

Posteriormente se mantiene 5 ciclos mas con un periodo de elongación de 7 min, finalmente esta se mantiene durante otros 7 min,



Una vez finalizados los ciclos de temperaturas programados, los tubos de PCR se mantienen a 4º C hasta que se realiza la electroforesis.

2.5. ELECTROFORESIS

2.5.1. Comprobación de la Integridad del ADN presente en los extractos.

a) Pesar una cantidad determinada de agarosa de tal forma que la concentración final sea del 0.8 % en solución 15 y añadir 7 µl de la solución 17.

b) Calentar la agarosa hasta que adquiera transparencia**.**

c) Enfriar ligeramente bajo el grifo y verter el contenido en el portageles con el peine incorporado.

d) Dejar polimerizar completamente la solución de agarosa.

e) Añadir 500 ng de ADN, 5 µl de marcador de peso molecular (Fago Lamda) y solución 16 en diferentes pocillos.

f) Sumergir el portageles en la cubeta que previamente se ha llenado con la solución 15 y conectar los electrodos en los extremos de la cubeta y regular la fuente de alimentación en 110 V

g) Dejar que el ADN migre al polo negativo durante media hora.

h) Comprobar el estado del ADN por comparación con el marcador de peso molecular.

2.5.2. Visualización de los Fragmentos Amplificados.

a) La visualización se realiza mediante electroforesis horizontal en geles de agarosa.

b) Pesar una cantidad determinada de agarosa (en función del tamaño del portagel donde se vaya a realizar la electroforesis) de tal forma

que la concentración final sea del 2.1% y proceder como en los apartados 1 a 4 del punto anterior.

c) Retirar el peine una vez polimerizada la agarosa y cargar en cada uno de los pocillos un volumen de 22 µl de la reacción de RAPD.

d) Dejar al menos 3 o 4 pocillos sin cargar volumen de reacción. En uno o dos de ellos añadir 2 µl de marcador de peso molecular, otro se utilizara como blanco (añadiendo igual volumen 22 µl que en las muestras) y en otro 3 µl de solución 16.

e) Proceder como en el apartado 6 anterior.

f) Dejar que los fragmentos de ADN migren hacia el polo positivo durante 4 h.

g) Pasado ese tiempo sacar la cubeta y colocar el gel en un trasiluminador. Grabar la imagen y analizar posteriormente el gel.

2.6. PROCEDIMIENTO DE ANÁLISIS.

a) Efectuar, al menos, dos extracciones diferentes de ADN en cada muestra a analizar.

b) Amplificar por duplicado y en diferentes días, cada uno de los extractos obtenidos.

c) Dentro de un mismo gel correr conjuntamente controles y expuestos, al menos de 3 a 4 reacciones de cada uno de ellos, junto con un marcador de peso molecular y una reacción amplificada sin ADN molde y considerada como blanco.

d) Para evitar la subjetividad del analista y una vez capturada la imagen por ordenador, analizar los perfiles fluorimetricos de cada uno de los gel mediante un software especifico, de forma cualitativa (ausencia o presencia de bandas) o de forma cuantitativa mediante parámetros específicos, tal como el porcentaje de amplificación.

ANEXO 2

PUBLICACIONES

ANEXO 2.1

CHARACTERIZATION OF RTG-2 FISH CELL LINE BY RANDOM AMPLIFIED POLYMORPHIC DNA. (1998)

***ECOTOXICOLOGY AND ENVIRONMENTAL SAFETY*. 40,** 56-64

Páginas 109 a 119

ECOTOXICOLOGY AND ENVIRONMENTAL SAFETY **40,** 56–64 (1998)
ENVIRONMENTAL RESEARCH, SECTION B
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Characterization of RTG-2 Fish Cell Line by Random Amplified Polymorphic DNA

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The increasing presence of genotoxic chemicals in the aquatic environment has led to the development of both *in vivo* and *in vitro* assays for target species. The fish population represents an important level of aquatic ecosystems that can be threatened by increased environmental pollution. The authors have studied the DNA pattern of the RTG-2 fish cell line, a fibroblast-like cell line, derived from rainbow trout (*Oncorhynchus mikyss*), to use this cell line as an *in vitro* system to study genotoxicity by means of random amplified polymorphic DNA primers (RAPDs). A constant pattern in the DNA band is essential when an organism or cell line is used to detect DNA alterations produced by genotoxic environmental chemicals. DNA fingerprints with RAPDs were obtained for RTG-2 by testing 26 single and 70 pairwise combinations of primers. Different methods of DNA extraction (chelating resin, salting out, and phenolization), the influence of spectrometric measures at 320 nm in the 260/280 quotient to quantify DNA extracts, genomic DNA and primer concentrations, annealing temperatures, and cell line passage were studied in the cell line characterization. RAPD products were identified by agarose gel electrophoresis. The good results obtained should allow the use of this system as a possible tool for detection of the genotoxicity of aquatic pollutants. © 1998 Academic Press

INTRODUCTION

The increasing concern over the presence of genotoxic compounds in the aquatic environments and its incidence in fish populations (Anderson *et al*., 1994; Wurgler and Kramers, 1992) have led to the development of new bioindicators of contaminant exposure and contaminant-induced effects on affected populations.

Interest in the actions of chemical mutagens in inducing DNA damage stems not only from the possibility that the presence of a chemical mutagen in the environment could result in an increased incidence of cancer, but also from the fact that exposure to these agents may result in an increased incidence of transmitted genetic disease (Evans, 1983).

An *in vitro* test using cells derived from homeothermic species is a powerful alternative to the use of live animals, in the first-tier genotoxicity test required for testing chemicals (L′OCDE guidelines 471 to 485). In addition, to avoid the use of vertebrates, the advantages of using *in vitro* assays are related mainly to cost, versatility, volume of waste, and laboratory facilities required. That, especially in the case of long-term fish bioassays, represents an important limitation for most conventional ecotoxicology laboratories.

The established fish cell line RTG-2, derived from rainbow trout (*Oncorhynchus mikyss*), has been successfully used in cytotoxicity research as an alternative system to acute fish bioassays (Bols *et al*., 1985; Babich *et al*., 1986; Castaño *et al*., 1994, 1996) and provides extremely useful results, in both cytotoxicity and genotoxicity studies, because this cell line maintains the ability to metabolize xenobiotics without the need of an exogenous metabolic system (Kocan *et al*., 1979, 1985a, b).

Genotoxicity assays, i.e. gene mutation, unscheduled DNA synthesis, chromosome analysis, sister-chromatid exchange, and micronucleus test, have been applied to fish cells (see Babich and Borenfreund, 1991, for a review), but the long cell cycle, the low capability of colony formation, and the large number and small size of their chromosomes make its routine use tedious and difficult. In recent years, molecular genetics has provided a number of powerful new methodologies that allow screening of the genomic structure of a given organism. One of the methodologies is random amplified polymorphic DNA (RAPD), developed by Williams *et al*. (1990). RAPDs result from a modification of the polymerase chain reaction (PCR) using arbitrary primers. RAPDs are new molecular markers for comparative genomic analyses that are quick and easy to use and practically unlimited in number.

0147-6513/98 $25.00

Because of their great simplicity, RAPDs have been found to be a powerful tool in the genomic map construction of different species (Ye *et al*., 1996; Faure *et al*., 1993), microorganism identification (Cocconcelli *et al*., 1995), taxonomical studies (Castiglione *et al*., 1993; Hu and Quiros, 1991), population studies (Welsh *et al*., 1991), and, recently, mutation detection (Kubota *et al*., 1995).

A consistent band pattern or fingerprint is essential and is the first step in the detection of genome alterations caused by environmental mutagens. These possible alterations can be expressed as a gain and/or loss of bands and variations in band intensit intensity (Kubota *et al*., 1995).

Extraction conditions, accurate quantification of the DNA template, concentrations and sequences of primers, annealing temperature, and stability of the cell line are factors that should be studied and defined, to obtain a reliable band pattern.

This paper presents the results of the study of these factors and the final protocol used to obtain a stable and specific fingerprint for the RTG-2 cell line. This band pattern allows exploration of the use of RAPDs in the detection of DNA alterations in environmental genotoxicology.

MATERIALS AND METHODS

RTG-2 cells (American Type Culture Collection, CCL 55) an established fibroblastic cell line derived from rainbow trout (*O. mykiss*), were grown in Eagle's minimum essential medium with Earle's salts (EMEM), supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 1.25 μg/ml fungizone, and 2 mM L-glutamine, in PVC tissue culture flacks (Costar) and incubated at $20 \pm 1^{\circ}C$ in a 5% CO_2/air atmosphere. Different line passages (100–135) were used.

Epithelioma papulosum cyprini (EPC) cells, an established epithelial cell line (Fijan *et al*., 1983) derived from carp (*Cyprinus carpio*), were grown in EMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 1.25 μg/ml fungizone, and L-glutamine 2 mM, in PVC tissue culture flacks (Costar), and incubated at $20 \pm 1^{\circ}C$ in a 5% CO_2/air atmosphere.

CHO-K1 cells (American Type Culture Collection, CCL 61), an established fibroblastic cell line derived from Chinese hamster ovary, were grown in Hams's F-12 medium supplemented with 10% fetal bovine serum and 10 μg/ml gentamicin, in PVC tissue culture flacks (Costar), and incubated at $37 \pm 1^{\circ}C$ in a 5% CO_2/air atmosphere.

Extraction Methods

The study of the extraction methods was carried out in six 75-cm^2 PVC flasks (Costar). Cells were dissociated with trypsin–EDTA (Flow, Scotland), collected in phosphate-buffered saline solution (PBS), divided into three aliquots, and centrifuged at 3000 rpm for 10 min.

Three different methods were used to isolate genomic DNA from the cell pellet: phenol extraction, high-salt precipitation, and boiling in the presence of a chelating resin.

Method I: Phenol extraction. DNA was isolated following a modification of the procedure of Sambrook *et al*. (1989).

About 2×10^6 cells were pelleted and then diluted in 0.5 ml of extraction buffer: 100 mM Tris–HCl, pH 8.0, 50 mM EDTA pH 8.0, 500 mM NaCl with 0.5% SDS (Dellaporta *et al*., 1983), and incubated at 65°C in a water bath for approx 15 min. To purify DNA, 165 μl 5 M potassium acetate was added and the mixture centrifuged at 12,000 rpm for 10 min; the supernatant was transferred to an Eppendorf tube and incubated with proteinase K and RNAse (Boehringer-Mannheim, Germany) at 37°C overnight. Digested proteins were extracted with Tris-buffered phenol, and the DNA (aqueous phase) was precipitated with isopropanol and then with 70% ethanol. DNA was diluted with sterile distilled water and its concentration estimated at 260 nm. Additional measures at 280 and 320 nm were performed.

Method II: Salting out. The procedure of DNA extraction and purification is the same as described for method I, but the extraction buffer used is 10 mM Tris–HCl and 2 mM Na_2EDTA, pH 8.2 (Miller *et al*., 1988). Phenol : chloroform extraction was replaced by high-salt precipitation of proteins with saturated NaCl (Cheng *et al*., 1995).

Method III: Chelating agent extraction. DNA was extracted using the Chelex chelating resin described by Walsh *et al*. (1991).

About 25,000 cells were pelleted at 3000 rpm for 4 min. The cell clamp was diluted with 0.45 ml of sterile distilled water, and 0.15 ml of 20% Chelex solution was added. After incubating at 56°C for 15 min, the suspension was vortexed and boiled for 2 min in a water bath. After a 10-min centrifugation at 12,000 rpm, the supernatant was transferred to a microcentrifuge tube, ready to be used for PCR amplification.

The sizes and integrity of the DNA fragments obtained in each extraction procedure were evaluated in 0.8% agarose gel electrophoresis.

DNA Amplification

Twenty-six primers ranging in size from 10 to 15 bases, with variable nucleotide proportion (G + C content), were designed, avoiding possible secondary structure formation. The purified oligonucleotides were supplied by the biopolymer department of CNBCR (I.S. Carlos III,

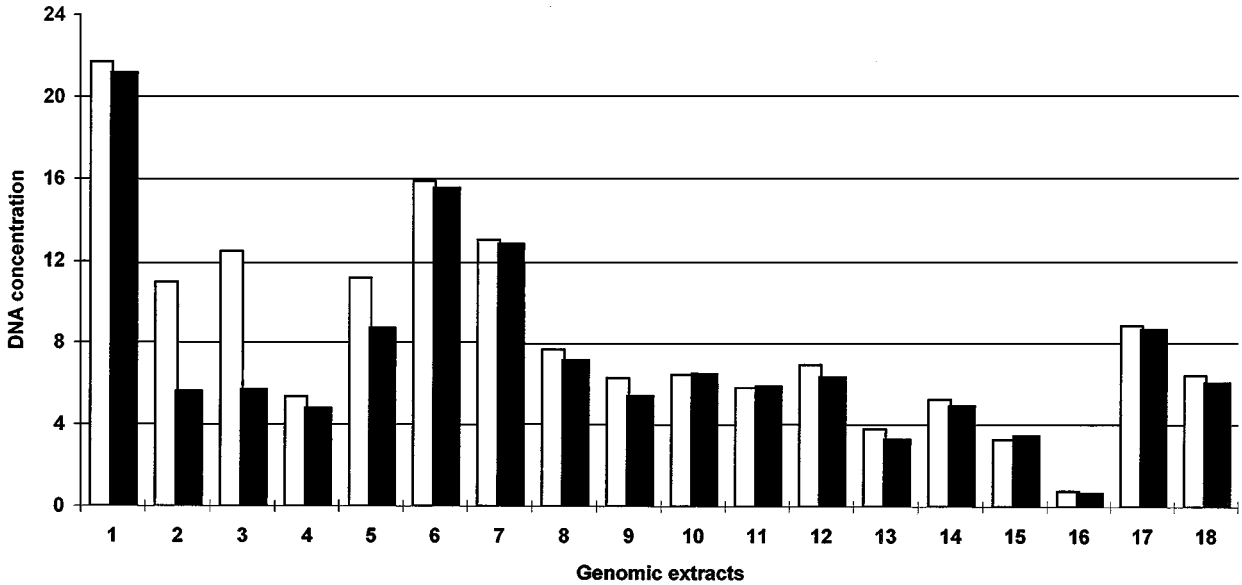

FIG. 1. Comparison of DNA concentrations of different genomic preparations with and without OD_{320} correction.

Majadahonda, Madrid). These 26 single primers and 70 pairwise combinations were tested for RAPDs.

Amplification was performed in 25-µl reaction volumes containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 4 mM $MgCl_2$, 0.2 mM of each dNTP (Pharmacia Biotech, Barcelona, Spain), and 2 U of Stoffel fragment (Perkin–Elmer). DNA template and primers were added at different concentrations ranging from 1 to 100 ng for DNA and from 1 to 8.5 pg for primers.

The RAPD protocol consisted of an initial denaturing step of 3 min at 92°C, followed by 45 cycles at 92°C for 25 s (denaturation), 36 or 39°C for 75 s (annealing), and 72°C for 6 min (extension). Five additional cycles were programmed at 92°C for 20 s, 36°C for 75 s, and 72°C for 7 min. Cycling was concluded with a final extension at 72°C for 7 min. The thermal cycler used was Perkin–Elmer Model 2400. All amplifications were done in duplicate and on different days.

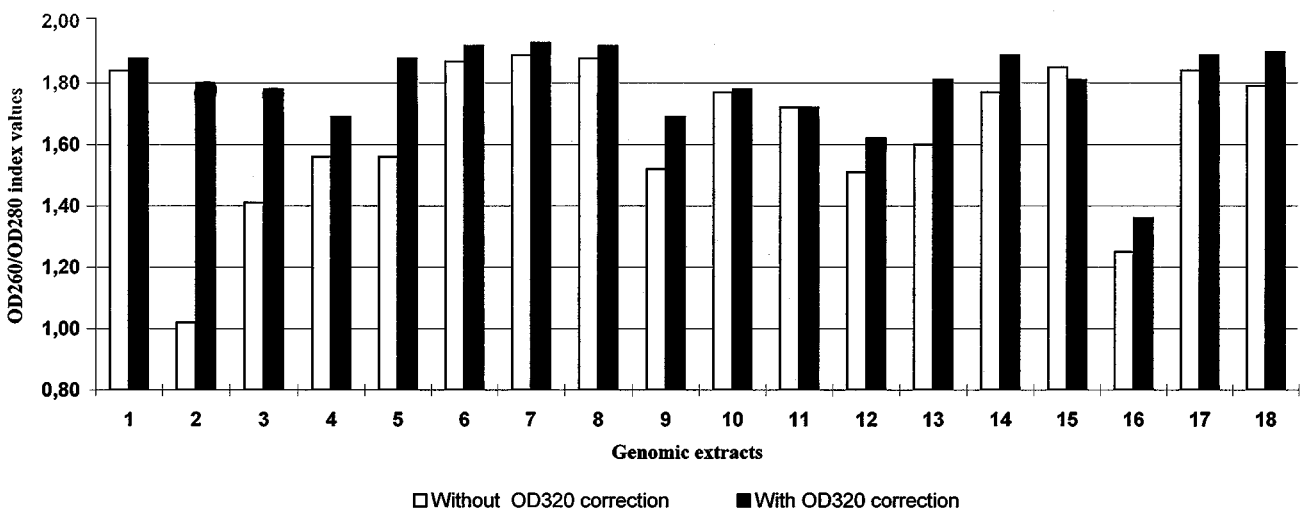

FIG. 2. Comparison of OD_{260}/OD_{280} ratio values of genomic preparations with and without OD_{320} correction.

TABLE 1
OD_{260}/OD_{280} Index Values in Extraction Comparative Assays

	Extraction		
Sample	Phenol : chloroform	Salting out	Chelex
1	1.68	1.73	3.21
2	1.83	1.8	3.44
3	1.8	1.86	3.17
4	1.83	1.82	3.23
5	1.86	1.9	3.76
6	1.73	1.82	3.07
Mean	1.79	1.82	3.31

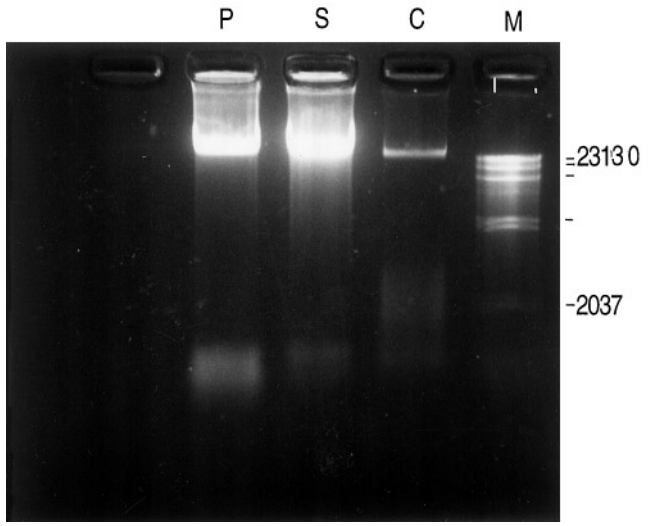

FIG. 3. Comparison of genomic DNA extracts. Aliquots of the same cell suspension were extracted by three different methods: phenol : chloroform (lane 1), salting out (lane 2), and Chelex resin (lane 3). Equal charges (approximately 1500 ng) of DNA from the three preparations were run on 0.8% agarose gel and stained with ethidium bromide. In all the three cases, genomic bands showed similar molecular weights (above 23.13 kb), although different fluorescene intensities were obtained. M, molecular weight marker.

Amplification products were separated electrophoretically in a 2.3% agarose gel using Tris–borate–EDTA (TBE) buffer system for 1 h at 115 V. Fractionated bands were detected by ethidium bromide fluorescence under UV light and photographed with a DS-34 Polaroid camera.

RESULTS AND DISCUSSION

The suitability of the extraction methods was evaluated on the basis of the extract purity, the integrity and efficiency of the genome amplification, and the yield obtained with each.

Concentration and purity of the DNA extract are usually measured at OD_{260} and by the 260 nm/280 nm absorbance ratio, but nonspecific background absorbance often disturbs these measurements (Muller *et al.*, 1993). An additional reading at 320 nm was performed to correct it. Figures 1 and 2 illustrate the influence of the 320 nm correction in the estimations of the DNA concentration and DNA purity of a set of 18 extracts.

While, in general, the values with and without the correction at 320 nm are very similar, exceptions exist that justify its use (extracts 2, 3, and 5). These extracts, looking at the 260/280 ratio, would have been rejected without the OD_{320} nm correction. Although these cases represent just a small percentage of the total, the systematic correction at OD_{320} measurement is recommended also to avoid DNA overestimation (Fig. 1), especially in the RAPD technique, where a great deal of precision is required because of the small amounts (15–2 ng) of DNA used.

The results of the purity grade (260/280 ratio after 320 correction) obtained for each extraction method are given in Table 1. Chelex extractions gave indexes above 3, whereas the values obtained with salting out and phenol : chloroform (1.68–2) indicated an acceptable grade of purity. In contrast, the yield obtained (0.1954 ng DNA/cell) using Chelex was

TABLE 2
DNA Yield in Extraction Comparative Assays

	Extraction		
Sample	Phenol : chloroform	Salting out	Chelex
1	0.001872	0.00361	0.1619
2	0.001042	0.00385	0.1488
3	0.000831	0.00247	0.1648
4	0.000551	0.00065	0.2375
5	0.000836	0.00058	0.1956
6	0.000404	0.00063	0.2637
Mean	0.000921	0.00196	0.1954

Note. Results are expressed as ng of DNA per cell.

TABLE 3
Primer Sequences Selected

Primer identification	Sequence	Length (bp)
D-4	5′-CTGTAGCATC-3′	10
D-8	5′-CCAAGTCGACA-3′	11
C-95	5′-CGGCCACTGT-3′	10
C-96	5′-AGCACTGTCA-3′	10
TRNA-1	5′-AGTCCGGTGCTCTA-3′	14
ALU-2	5′-GACCCGCACC-3′	10

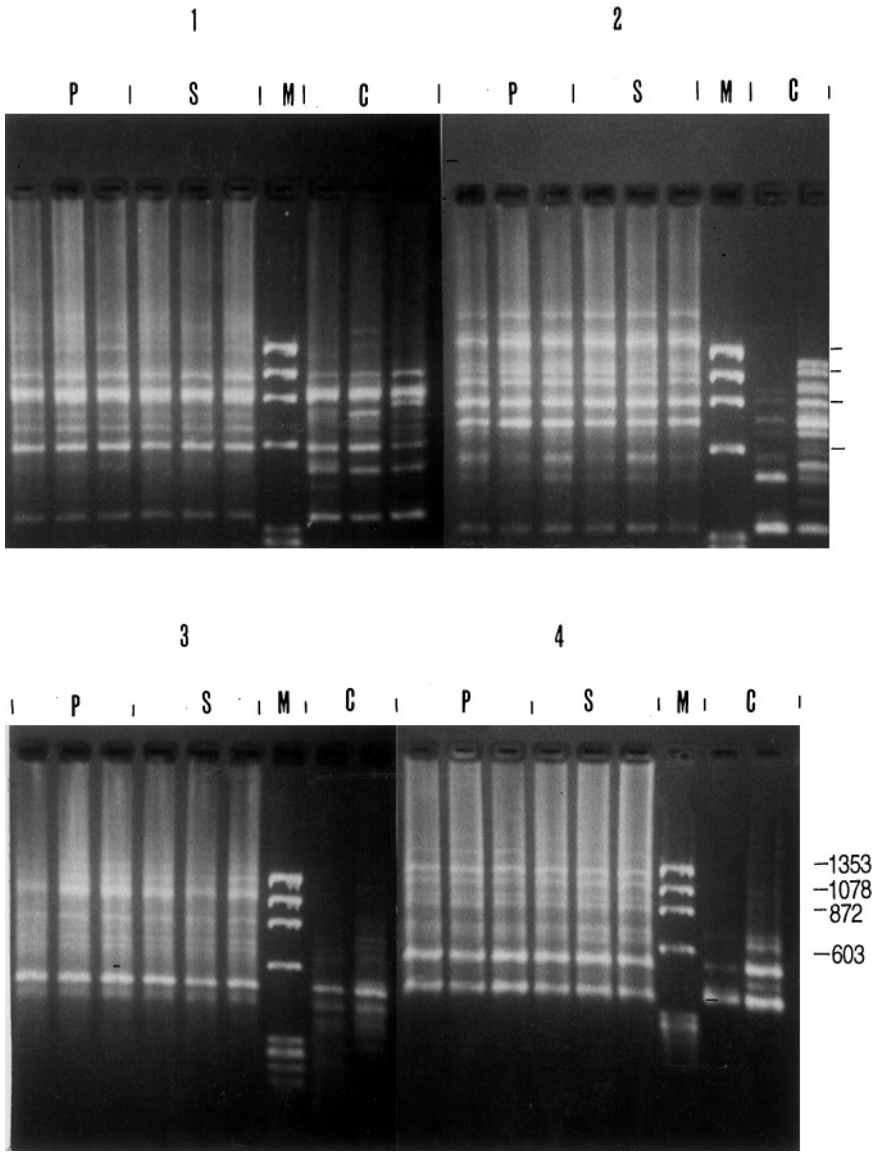

FIG. 4. Comparison of purified. DNA efficiency in RAPD reaction. DNA templates obtained from the three extraction procedures were amplified with T-1 (1), D-8 (2), C-95 (3), and C-96 (4) primers. P, S, and C lanes correspond to phenol : chloroform, salting out, and Chelex extraction procedures, respectively. Chelex extracts gave patterns inconsistent with those obtained by the other two methods. M, molecular weight marker.

about 200% higher than the yields of the other two methods (Table 2). Salting out and phenol: chloroform methods gave similar results.

The integrity of the genomic DNA extracted by each procedure from the same charged amount of DNA is illustrated in Fig. 3. The electrophoresis indicates that the sizes of the genomes obtained by the three methods are similar, but the fluorescence intensity of the Chelex extract is considerably lower than those of the other two extracts. There are no differences in fluorescence intensity between the salting out and phenol : chloroform methods.

The effectiveness of the different methods was evaluated by studying the amplification of each of the genomic extracts. The band patterns obtained with phenol : chloroform and salting out (Fig. 4) are the same and highly reproducible in all cases, in contrast with the band pattern obtained with Chelex.

The presence of RNA as an impurity in Chelex extracts would justify the results obtained: values above 2 in the OD_{260}/OD_{280} index and erroneous calculation of the DNA concentration. A lack of precision in genome quantification, caused by the undetected presence of RNA in the

electrophoresis or partial destruction of the same, could have led to the nonrepeated results with the same and/or with different extracts.

The use of Chelex resin in PCR studies gives good results because the aim of the study is the amplification of a precise sequence (Walsh *et al*., 1991). However, in the scanning of a complete genomic DNA pool (RAPDs technique), this extraction method is not recommended.

Band Pattern

The selection of the primers to obtain a specific and stable band pattern, as well as the amplification conditions, depends on the number of bands obtained with each and their intra- and intergenomic repeatebility.

An annealing temperature of 36°C gives better results than 39°C for most of primers tested. Although amplification samples can be obtained in a wide range of DNA and primer concentrations, each oligonucleotide provides an optimum value depending on the number of bands obtained. For example, using D-4, which provides only one band, the best amplification is obtained with 2 ng of genome and 2 pmol of primer. With respect to the best general conditions for the primers that give better results (large and constant number of bands), an annealing temperature of 36°C, 15 ng of DNA, and 5.0 pmol of primer were chosen.

Amplifications with single primers gave better results than the binary combinations tested, in some cases because of the failure of amplification, lack of intergenomic reproducibility, appearance of bands with the same molecular weight as the single primers, or polymerization of the amplified products.

Of the 26 single primers tested, only 6 gave reproducible results. The primers selected for the band pattern were oligonucleotides from 10 to 14 bases (Table 3), which generated a number of bands above 40, from 300 to 1500 bp in size.

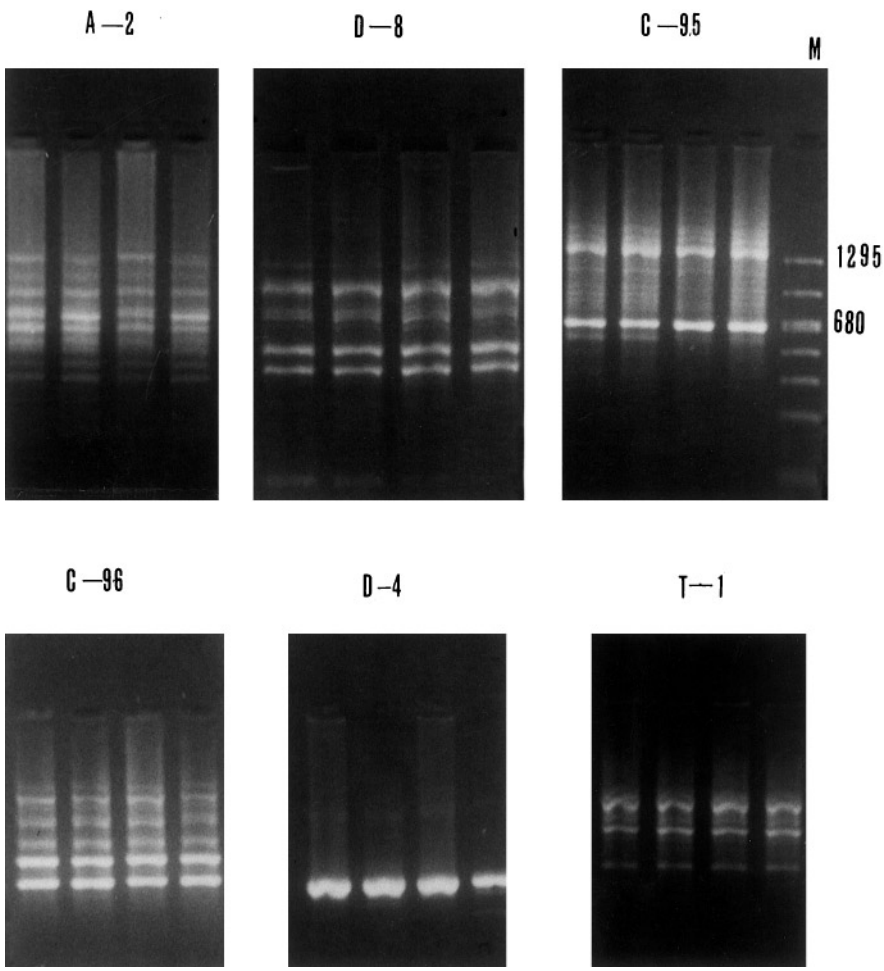

FIG. 5. Fingerprint obtained for RTG-2 cell line. RAPD pattern generated by the chosen primers A-2, D-8, C-95, C-96, D-4, and T-1 proved to be stable for a set of different RTG-2 genomic extracts. M, molecular weight marker.

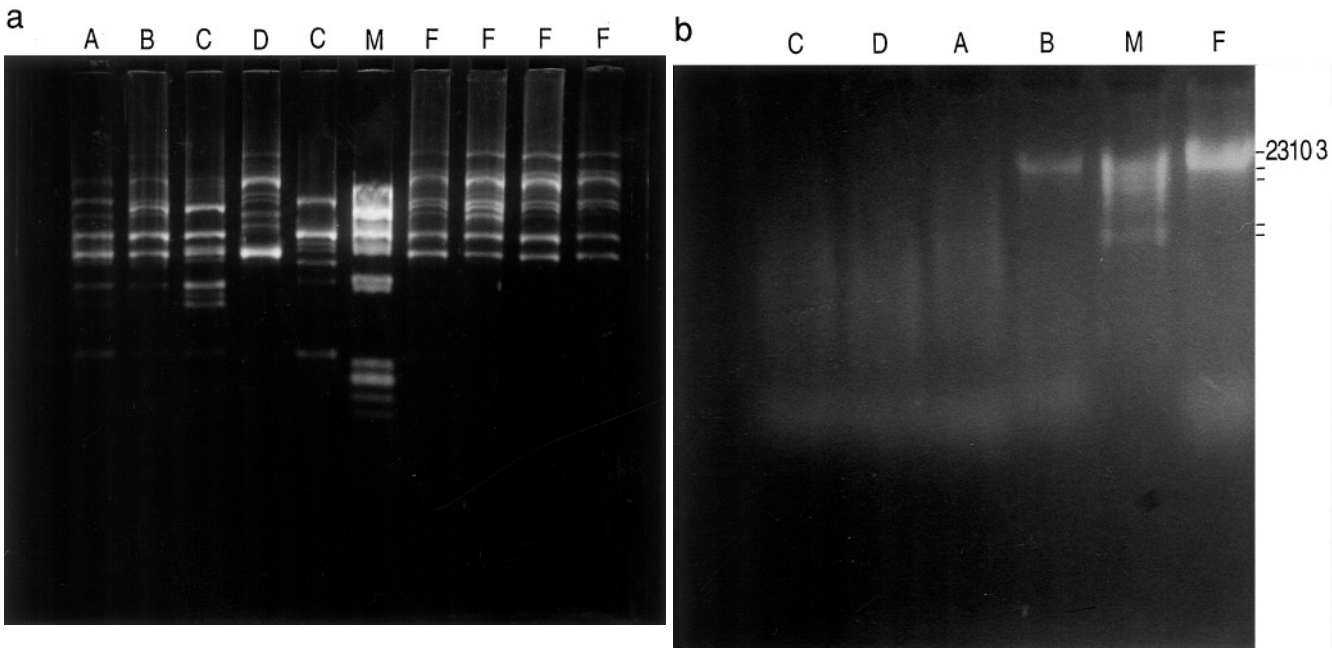

FIG. 6. Effect of DNA template integrity on the reproducibility of the pattern. (a) The D-8-specific DNA profile shown in F is not reproduced by genomes A, B, C, and D. (b) The same amounts of genomic extracts that failed to give the amplification pattern expected were loaded in 0.8% agarose gel to check their integrity. No discrete genomic band was visualized by C, D, and A, and a loss in fluorescence intensity is observed in B when compared with genomic extract F. M, molecular weight marker.

Under the selected conditions the fingerprint obtained was stable and specific (Fig. 5). The occurrence of intraassay variations (Fig. 6a) has been attributed to failures in the extraction procedure and/or genomic degradation (Fig. 6b).

In the amplifications carried out with genomic extracts derived from different cell passages (from 100 to 135), no differences were observed in the band patterns. The specificity of the pattern was tested by amplifying genomes extracted from the EPC and CHO-K1 cell lines with four of the six selected primers (C-95, C-96, D-4, Trna-1) (Fig. 7). Results obtained reflect differences between RTG-2 fingerprint and both EPC and CHO-K1 fingerprints. Differences were also found between the two fish cell lines (RTG-2 and EPC cells) and the mammal cell line (CHO-K1): thus, D-4 primer provides products of equal molecular weight in the three cell lines studied; another product, of high molecular weight, only in the mammalian line (CHO-K1 cells), may be a consequence of its different position on the evolutionary scale.

Based on the usefulness of the RAPD technique for the differentiation of very close populations and the detection of mutations produced by radiation of medaka eggs (Kubota *et al*., 1995), the purpose of obtaining a specific band pattern for the RTG-2 cell line (Fig. 5) is to use this *in vitro* system as a tool for the detection of environmental DNA damage in fish. A loss or gain in bands as well as densimetric differences in them would be interpreted as an alteration in genomic DNA. Preliminary results using mitomycin C as reference mutagen (data not provided) left us optimistic about this point, although future research is needed to confirm its usefulness in aquatic genotoxicology.

CONCLUSIONS

The OD_{320} correction, in the establishment of purity grade and DNA amount in an extract, is not usual practice, but the results obtained with genomic extracts would make it advisable on a routine basis in the RAPD technique.

Phenol : chloroform and salting out extraction methods are suitable for use in the RAPD technique because of the grade of purity, integrity, and effectiveness obtained. Although the yield obtained with Chelex extraction is higher, the results do not recommend its use.

The band pattern established using primers D-4, D-8, C-95, C-96, Trna-1, and Alu-2 is specific to and stable in the RTG-2 cell line. It is formed by a number of bands above 40 (between 300 and 1500 bp).

The results of preliminary studies carried out in the laboratory using mutagenic products such as mitomycin C allow optimism in the use of this technique for the detection of mutations induced by the presence of chemical mutagens in the aquatic environment.

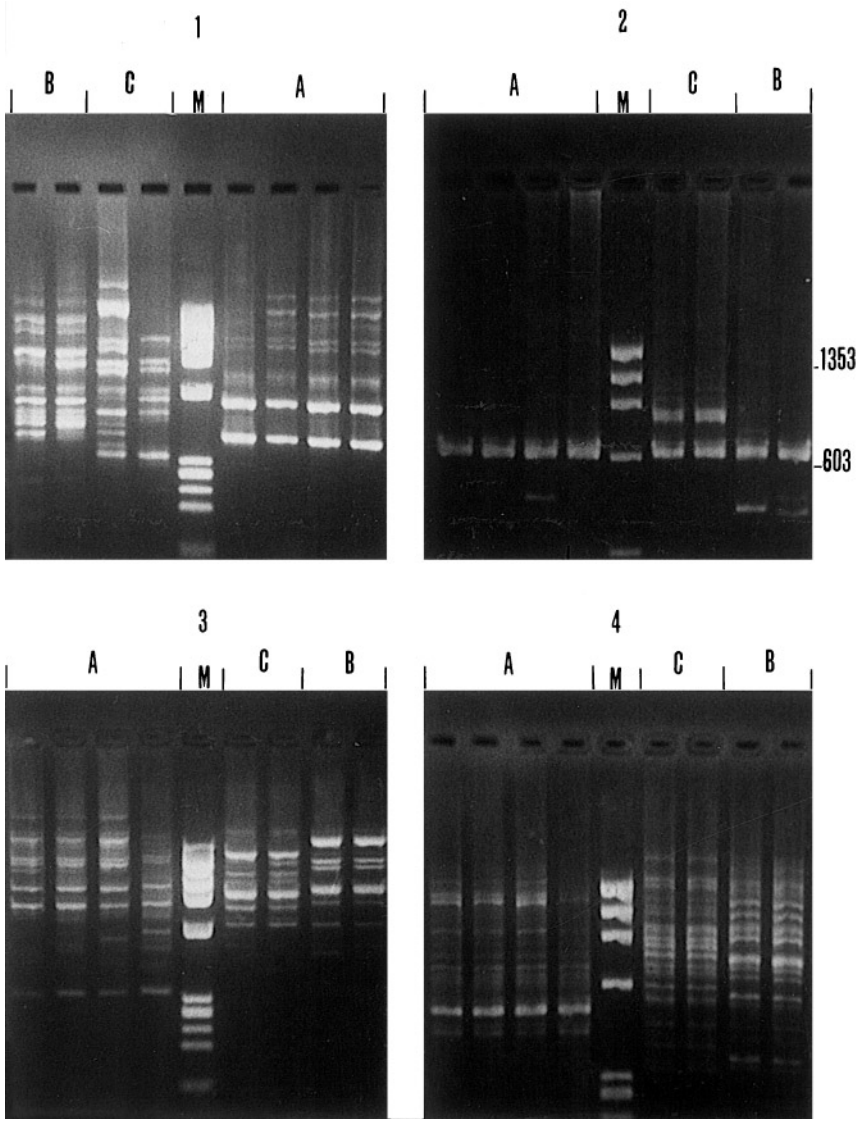

FIG. 7. Amplification of DNA from different species. RAPD analysis with C-96 (1), D-4 (2), D-8 (3), and T-1 (4) primers under fixed conditions was performed over DNA isolated from RTG-2, EPC, and CHO-K1 cell lines (lanes A, B, and C respectively). Different random products were obtained for each cell line. M, molecular weight marker.

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ANEXO 2.2

DETECTION OF MITOMYCIN C-INDUCED GENETIC DAMAGE IN FISH CELLS BY USE OF RAPD. (1999)

***MUTAGENESIS* VOL. 14, Nº 5, 449 – 456,**

Páginas 121 a 130

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Detection of mitomycin C-induced genetic damage in fish cells by use of RAPD

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Concern about genetic alterations in fish populations arising from anthropogenic activities has led to the adaptation and/or development of new tests and techniques that shed light on these alterations. The high number and the reduced size of chromosomes and the long cell cycle associated with most fish species preclude the use of most accepted genotoxicity assays. The purpose of this work was to study the capability of the randomly amplified polymorphic DNA technique to show genotoxic effects induced by chemicals in fish cells. To do that we studied the effect of 0.5 μg/ml mitomycin C (MMC) on an established rainbow trout cell line (RTG-2). To increase the sensitivity of detecting altered copies of DNA and to avoid the presence of false positives and a lack of reproducibility, the amounts of DNA template and primer present in amplification reactions were studied and optimized after comparison between the control and exposed fingerprints for 4, 6 and 8 h. Results show that 5 ng of DNA template and 4 pM chosen primer were optimum to show differences between control and exposed cells and to obtain reproducible results. The results obtained, after optimum conditions were established, show that this system could be useful for the assessment of DNA alterations in *in vitro* genotoxicity studies.

Introduction

Sublethal exposure to environmental genotoxic agents arising from human activity causes different forms of alterations to exposed aquatic populations, which may lead to the structure and function of ecosystems being altered (Anderson *et al*., 1994).

These derived effects of genotoxins directly or indirectly affect genome integrity due to mutations in germinal and/or somatic cells, leading to an increase in the incidence of different types of tumours (Leblanc and Bain, 1997) and, in the long term, to alterations in the genetic variability of the exposed populations (Theodorakis *et al*., 1998).

These facts, and for risk assessment purposes, make it imperative that prior laboratory tests are carried out to establish if a particular pollutant or a complex mixture can cause adverse affects on the genetic material of the exposed populations before its liberation into the aquatic environment.

However, reproducing the effects on a fish population of long-term exposure to genotoxic agents, or even short-term exposure but with long-term expression of the effects, requires the availability of large laboratory facilities, personnel specialized in maintaining the species and a large volume of samples, all of which make it very costly.

In vitro tests using fish cell lines are an alternative to the use of fish and have big advantages in ecotoxicology studies (Babich and Borenfreund, 1991; Castaño *et al*., 1994). The RTG-2 cell line derived from rainbow trout (*Oncorhynchus mikiss)* has been widely used in cytotoxicity studies and shows a good correlation with *in vivo* acute fish bioassays (Bols *et al.*, 1985; Castaño *et al.*, 1996). Owing to its capacity to metabolize xenobiotics without requiring the addition of metabolic activation systems (Smolarek *et al*., 1988; Araujo *et al.*, 1998), it has also been shown to be appropriate for genotoxicity studies (Kocan *et al*., 1985).

The OECD *Guidelines* (1998) list a series of widely used techniques, both *in vivo* and *in vitro*, for use in risk assessment procedures associated with genotoxins for humans. However, the application of these traditional techniques to the detection in DNA of both macro- and micro-damage (sister chromatid exchange, chromosomal aberrations, etc.) are particularly tedious when applied to fish, due to the high number of chromosomes and the long cell cycle found in fish cells. The appearance of new assays in the last few years, such as the Comet assay (McKelvey *et al.*, 1993; Pandangi *et al.*, 1996), automatic scoring techniques for micronuclei assays (OCDE, 1998) and ^{32}P-post-labelling for the detection of adducts (Phillips *et al*., 1997), together with recent advances in molecular biology, such as DNA fingerprinting and gene amplification by PCR, offer new possibilities for detecting DNA damage, circumventing these limitations.

Randomly amplified polymorphic DNA (RAPD), developed by Williams *et al.* (1990), is a technique that involves the amplification of random segments of genomic DNA using the PCR methodology. This method does not require prior sequence information and arbitrarily chosen short primers are used at low stringency to amplify multiple segments from genomic DNA to any species (Williams *et al.*, 1990). The majority of such fragments are identical between individuals or strains, which represents a tremendous potential for application in different research fields: the study of genetic variation in natural populations (Welsh and McClelland, 1991; Theodorakis *et al.*, 1998), genetic variability studies (Paffeti *et al*., 1996; Endtz *et al.*, 1997) and mutation detection in *in vivo* studies (Kubota *et al*., 1992, 1995; Theodorakis and Shugart, 1997; Atienzar *et al*., 1998).

In the same way, cell populations exposed *in vitro* to genotoxins suffer DNA alterations in a certain number of cells, which are reflected as variations in the fingerprint obtained for the control population. These are defined as band losses and/or gains as well as alterations in the intensity of amplification of some of them. Such alterations *in vivo* are considered mutations that are produced by changes to, deletions of or insertions into the pair bases (Muralidharan and Wakeland, 1993).

In a previous work, the characteristic fingerprint of the RTG-

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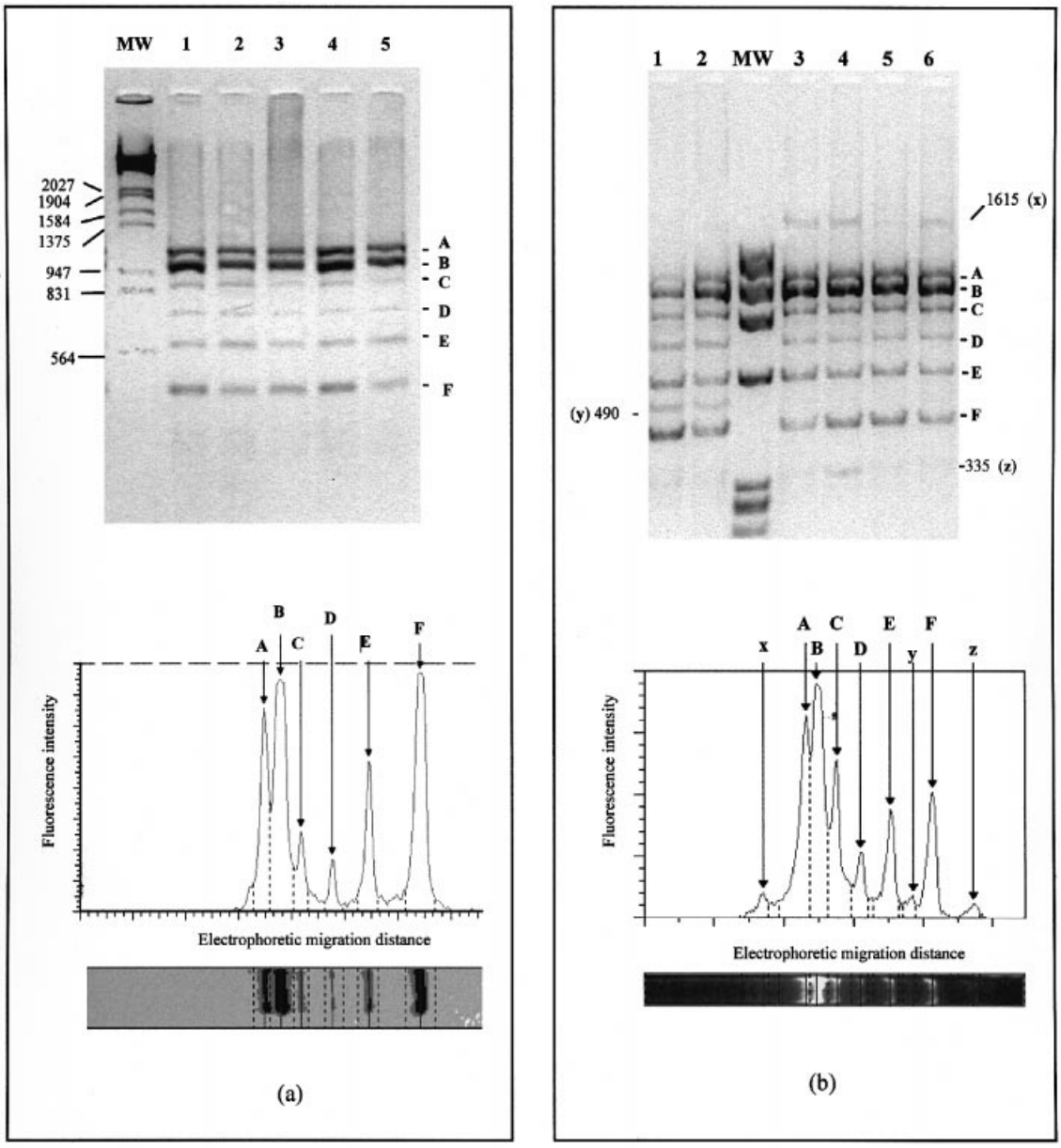

Fig. 1. Amplification and fluorescence profiles of band pattern generated by the AA-82 primer at two template concentrations. (**a**) Amplifications carried out with 5 ng of genomic DNA. (**b**) Amplifications carried out with 20 ng of genomic DNA; bands x, y and z of 1615, 490 and 340 bp, respectively, were considered erratic bands. The RTG-2 fingerprint with this primer was established with the six common bands: three of high molecular weight, bands A–C (1075, 965 and 840 bp, respectively); one of medium molecular weight, band D (700 bp); two of low molecular weight, bands E and F (570 and 430 bp, respectively).

2 cell line was established in order to use this methodology for the detection of DNA damage after exposure of cell populations to genotoxic agents (Ferrero *et al.*, 1998).

The RAPD technique has been associated with some limitations concerning lack of reproducibility and the appearance of 'ghost' bands which are difficult to interpret. In this work we have studied some variables in order to avoid these limitations. For that, a well-known clastogenic agent was used, mitomycin C (MMC), an inducer of DNA interstrand crosslinks (Sehlmeyer *et al.*, 1996), DNA adducts and mutations (Sanderson and Shield, 1996), both *in vivo* (Fahring, 1977; Ehling, 1978; Cao *et al.*, 1993; Grawé *et al.*, 1993) and *in vitro* (Kato and Shimada, 1975; Singh and Gupta, 1983; Davies *et al.*, 1993; Salvadori *et al.*, 1994).

To increase the sensitivity of detection of altered copies of DNA and to avoid the presence of false positives and lack of reproducibility, the amounts of DNA template and primer present in amplification reactions were studied and optimized after comparison between the control and exposed fingerprints for different exposure periods. The final purpose of this work was thus to minimize the limitations of this technique in order to be able to apply it to the detection of genomic DNA alterations caused by environmental chemicals to exposed cells.

Materials and methods

Cell cultures

RTG-2 cells (CCL 55; American Type Culture Collection, Rockville, MD), an established fibroblastic cell line derived from rainbow trout (*Oncorhynchus mykiss*) were grown in Eagle's minimum essential medium with Earle's salts (EMEM), supplemented with 10% fetal serum, 100 U/ml penicillin,

Table I. Frequency of appearance of each of the bands obtained with 5 and 20 ng of genomic DNA

Band	Size (bp)	5 ng (%)	20 ng (%)
x	1615	0	61
A	1075	100	100
B	965	100	100
C	840	100	100
D	700	84	94
E	570	100	100
y	490	0	4
F	430	100	100
z	335	0	14

Bands A–F form the fingerprint control. x, y and z are erratic bands. Twenty-three amplifications (two genomic DNA extracts) were carried out at both template concentrations. The data obtained is the result of assigning the value 1 (presence) or 0 (absence of band) to each band in each amplification reaction.

Table II. Differences in frequency of appearance of bands between controls and exposed cells at 4, 6 and 8 h: (**a**) 5 ng of genomic DNA; (**b**) 20 ng of genomic DNA

Band	Size (bp)	Control	4 h	6 h	8 h
a					
A0	1345		5	0	0
A	1075	100	100	100	85
B	965	100	95	100	93
C	840	100	90	100	90
C1	780		0	0	7
D	700	84	50	40	40
D1	645		1	5	21
E	570	100	95	100	91
E1	545		0	0	2
F	430	100	100	100	100
F1	410		0	5	2
b					
A0	1345		0	0	0
A	1075	100	100	100	100
B	965	100	100	100	100
C	840	100	100	100	100
C1	780		0	0	0
D	700	84	100	75	75
D1	645		0	0	0
E	570	100	100	100	88
E1	545		0	0	0
F	430	100	100	100	100
F1	410		0	0	0

Data are expressed as percentages. Numbers of replicate reactions were as follows: (a) Control, 23; 4 h, 20; 6 h, 20; 8 h, 36 and (b) control, 23; 4 h, 8; 6 h, 8; 8 h, 8.

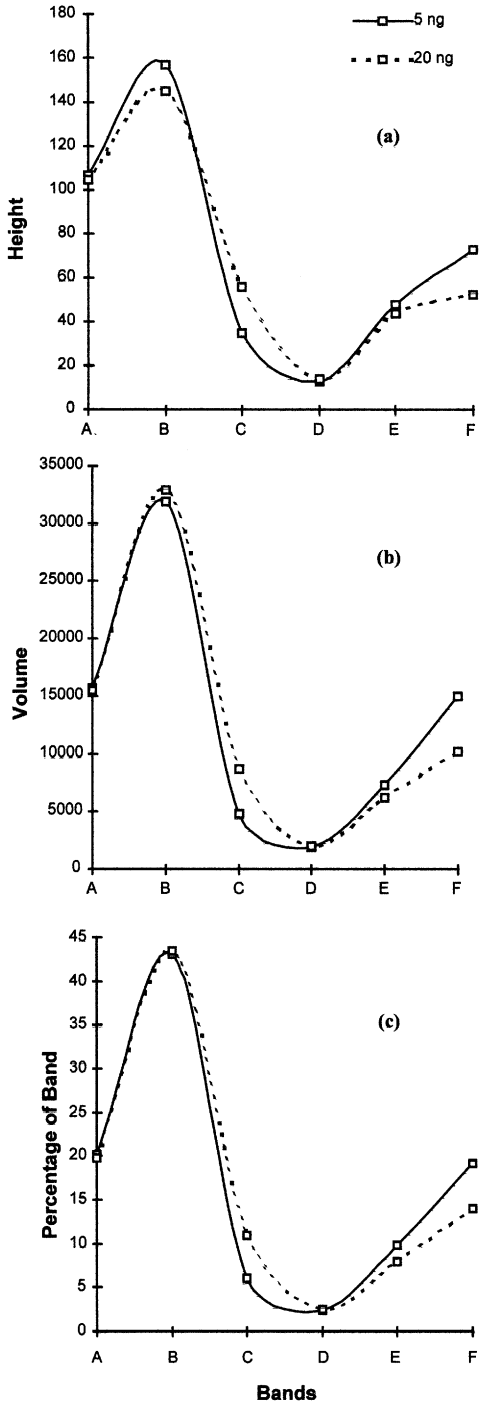

Fig. 2. Graphic representation of quantitative differences in the RTG-2 fingerprint using 5 and 20 ng of template. In both cases bands A, B, D and E coincide and bands C and F are directly and inversely proportional to the genomic DNA concentration. (**a**) Height. (**b**) Volume. (**c**) Percentage of band amplified.

100 μg/ml streptomycin, 1.25 μg/ml fungizone and 2 mM L-glutamine, in PVC tissue culture flasks (Costar, Corning, NY) and incubated at 20 ± 1°C in a 5% CO_2/air atmosphere.

Exponentially growing cells were exposed to 0.5 μg/ml MMC (Sigma, St Louis, MO) for 4, 6 or 8 h. After each exposure period, the medium was removed and the cells washed with phosphate-buffered saline (PBS). Fresh medium was then added and the cells were left to grow for a further 72 h (~1.5 cell cycles).

Two 75 cm^2 flasks were used for the control and for each one of the exposure periods. The experiment was repeated twice on different days.

DNA extraction and RAPD reaction

Cells were dissociated with trypsin–EDTA (Flow Laboratories, Rickmansworth, UK), collected in PBS, pH 7.3, and their DNA isolated by phenol extraction (Ferrero *et al*., 1998). The integrity of the extracted genomic DNA was checked by 0.8% agarose electrophoresis using the λ phage as molecular weight marker (Eurobio, Paris, France). Amplification was performed in a 25 μl volume containing buffer solution, 4 mM $MgCl_2$, 2 U Stoffel fragment (Perkin-Elmer, Branchburg, NY) and 0.2 mM each dNTP (Pharmacia, Barcelona, Spain). DNA template was added at two different concentrations, 20 and 5 ng.

The RAPD protocol was previously described by Ferrero *et al*. (1998). Briefly, an initial denaturing step at 92°C was followed by 45 cycles of annealing at 36°C for 75 s and extension at 72°C for 6 min. The thermal cycler used was a Perkin-Elmer model 2400.

The primer used was AA-82 (5′-GATCCATTGC-3′; Biopolymers Department, CNBCR, I.S. Carlos III, Madrid, Spain). This primer was selected

Table III. Results of quantitative analysis of height, volume and percentage of amplified bands with 5 ng of genomic DNA for controls and exposed cells

		Height	Volume	Percentage of band	n
Band A	Control	107 ± 26.3	15741 ± 4555	20.0 ± 4.7	23
	4 h	89 ± 28.4	12707 ± 5714	20.8 ± 9.9	20
	6 h	69 ± 32.7[c]	9980 ± 5157[b]	14.1 ± 6.9[b]	20
	8 h	80 ± 26.8[b]	10620 ± 3919[c]	12.2 ± 4.8[c]	35
Band B	Control	157 ± 26.7	33227 ± 9785	43.1 ± 8.8	23
	4 h	162 ± 10.9	32543 ± 7841	46.1 ± 6.0	20
	6 h	107 ± 33.7[c]	19841 ± 6608[c]	28.4 ± 8.7[c]	20
	8 h	145 ± 39.2	27445 ± 9428[a]	29.6 ± 10.4[c]	36
Band C	Control	35 ± 14.9	4887 ± 2503	6.1 ± 2.7	23
	4 h	52 ± 19.8[b]	7328 ± 3019[a]	8.9 ± 3.4	18
	6 h	23 ± 10[a]	3446 ± 1871	4.7 ± 2.4	20
	8 h	20 ± 9.4[b]	2924 ± 1411[b]	3.0 ± 1.1[c]	36
Band D	Control	13 ± 4.9	1872 ± 781	2.4 ± 1.0	19
	4 h	19 ± 12.5	2694 ± 1854	3.9 ± 2.4	10
	6 h	9 ± 3.0	1565 ± 513	2.2 ± 0.6	8
	8 h	27 ± 20.9[a]	4879 ± 3644[c]	5.3 ± 4.2[b]	23
Band E	Control	48 ± 16.9	7258 ± 2691	9.8 ± 3.1	23
	4 h	33 ± 16.9[a]	4929 ± 2773[b]	7.4 ± 3.6	19
	6 h	55 ± 33.1	8877 ± 5333	13.0 ± 8.0	20
	8 h	67 ± 38.4	12668 ± 6934[b]	13.9 ± 6.4[b]	35
Band F	Control	73 ± 33.6	14459 ± 6750	19.2 ± 10.3	23
	4 h	38 ± 24.2[c]	6970 ± 4876[c]	13.0 ± 10.3[a]	20
	6 h	123 ± 46.7[b]	27187 ± 12304[b]	38.6 ± 16.7[c]	20
	8 h	116 ± 50.2[b]	27452 ± 12446[c]	35.9 ± 20.9[c]	36

Values are means ± SD of *n* amplifications of different genomic DNA extracts (when the band was not present, it was given no value). Significant differences versus control, [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.0001$ by Mann–Whitney *U*-test.

Table IV. Results of quantitative analysis of height, volume and percentage of amplified bands with 20 ng of genomic DNA from control and exposed cells

		Height	Volume	Percentage of band	*n*
Band A	Control	104.7 ± 27.1	15484 ± 5558	19.8 ± 4.8	23
	4 h	100.9 ± 30.5	16537 ± 6113	20.7 ± 5.9	8
	6 h	78.0 ± 23.8	11669 ± 4246	20.0 ± 5.9	8
	8 h	110.3 ± 24.3	15230 ± 3961	19.9 ± 4.6	8
Band B	Control	145.1 ± 13.4	32932 ± 7626	43.5 ± 5.3	23
	4 h	156.0 ± 11.7	37658 ± 7492	46.2 ± 4.4	8
	6 h	139.8 ± 22.8	28702 ± 7338	45.0 ± 5.4	8
	8 h	145.8 ± 22.1	35409 ± 8124	47.6 ± 3.5	8
Band C	Control	55.9 ± 10.2	8689 ± 1577	11.0 ± 2.1	23
	4 h	55.0 ± 11.6	7718 ± 1938	10.0 ± 2.4	8
	6 h	42.0 ± 8.2[b]	6398 ± 1668[b]	11.3 ± 3.1	8
	8 h	59.0 ± 17.8	9155 ± 4259	11.6 ± 3.4	7
Band D	Control	13.8 ± 3.8	2032 ± 635	2.5 ± 0.8	21
	4 h	14.6 ± 5.4	2303 ± 1195	2.9 ± 1.1	8
	6 h	11.0 ± 1.8	1548 ± 220	2.8 ± 0.8	6
	8 h	21.3 ± 4.5[b]	2842 ± 431[b]	2.6 ± 1.6	6
Band E	Control	43.6 ± 11.3	6242 ± 1536	8.0 ± 1.8	23
	4 h	44.3 ± 11.2	7146 ± 3058	9.6 ± 3.5	8
	6 h	33.0 ± 6.8[a]	4948 ± 1888	8.7 ± 3.5	8
	8 h	39.9 ± 12.4	5327 ± 1924	8.2 ± 2.1	7
Band F	Control	52.4 ± 23.4	10166 ± 3686	14.0 ± 5.8	23
	4 h	30.6 ± 9.4[a]	6367 ± 2320	9.8 ± 4.4	8
	6 h	22.8 ± 6.2[b]	4429 ± 1663[b]	12.1 ± 5.5	8
	8 h	48.5 ± 17.0	7513 ± 3263	10.2 ± 3.5	8

Values are means ± SD of *n* amplifications of different genomic DNA extracts (when the band was not present, it was given no value). Significant differences versus control, [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.0001$ by Mann–Whitney *U*-test.

because with this cell line it generates different amplification products within a wide range of molecular weights.

Amplification products (22 µl) were resolved electrophoretically on 2.1% agarose gels and stained with ethidium bromide. The image was recorded by the Grab-it program (UVP, Uplad, CA).

Optimization of primer concentration

In order to avoid the presence of artefacts, we carried out 20 amplifications on different days, without DNA template and using three different primer concentrations (2.5, 4 and 5 pM) following the above described amplification conditions.

Table V. Grouped values of height, volume and percentage of amplified bands with 5 and 20 ng of template from control and exposed cells according to molecular weight

		Height	Volume	Percentage of band	*n*
Band of high molecular weight (>800 bp)					
20 ng	Control	306 ± 42.5	57105 ± 12278	74.3 ± 7.9	23
	4 h	312 ± 49.0	61913 ± 13869	76.9 ± 8.3	8
	6 h	260 ± 50.4[a]	46769 ± 10991[a]	76.3 ± 8.1	8
	8 h	315 ± 58.3	59794 ± 14111	79.1 ± 3.9	8
5 ng	Control	298 ± 58.5	53855 ± 14110	69.2 ± 11.4	23
	4 h	304 ± 51.6	52578 ± 13828	75.8 ± 6.1[b]	20
	6 h	199 ± 71.9[c]	33266 ± 12342[c]	47.1 ± 16.5[c]	20
	8 h	279 ± 70.8	47307 ± 13766	44.8 ± 13.4[c]	36
Band of low molecular weight (<600 bp)					
20 ng	Control	96 ± 33.5	16408 ± 4709	22.0 ± 7.5	23
	4 h	75 ± 22.8	13513 ± 5013	19.4 ± 7.7	8
	6 h	56 ± 15.7[b]	9377 ± 3357[b]	20.8 ± 7.6	8
	8 h	88 ± 31.7	12840 ± 4789	18.4 ± 1.9	8
5 ng	Control	80 ± 26.4	21716 ± 7601	29.0 ± 9.0	23
	4 h	70 ± 23.1[c]	11898 ± 4200[c]	20.4 ± 7.2[b]	20
	6 h	178 ± 52.9[c]	36063 ± 12153[c]	51.5 ± 16.4[c]	20
	8 h	183 ± 74.6[b]	40120 ± 15326[c]	49.8 ± 17.9[c]	36

Values are means ± SD of *n* amplifications. Significant differences versus control, [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.0001$ by Mann–Whitney *U*-test.

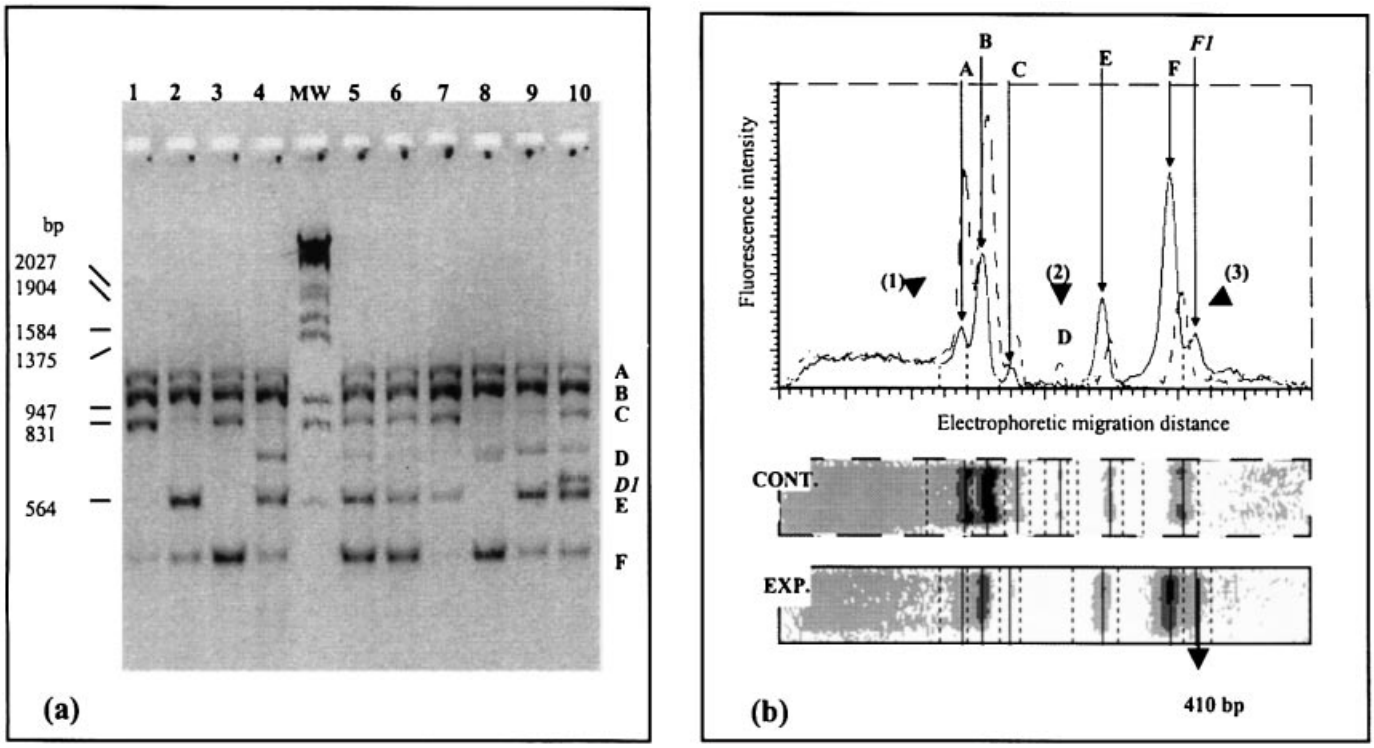

Fig. 3. Differences observed between genomic DNA of control and exposed cells. (**a**) Amplifications carried out with 5 ng of DNA from control and exposed cells. Control fingerprint (lines 4–6) bands A–F. The fingerprints of exposed cells (lines 1–3 and lines 7–10) show alterations such as absence of bands (lines 1, 3 and 8, band E) or the presence of other new bands (line 10, band D1). (**b**) Comparison of the fluorimetric profile of two amplifications carried out with DNA from control (dotted line) and exposed (solid line) cells where three types of differences are observed: (1) different intensity of amplification; (2) absence of bands (loss of band D); (3) presence of a new band (band F1, of 410 bp).

Analysis of the band pattern of control and exposed cells

The genomic DNA extract in the two different flasks was obtained and amplified at least twice on different days. The control and exposed cells for each experiment were individually amplified but developed together in the same gels, using the molecular weight markers ϕX174-*Hae*III and λ/*Eco*RI-*Hin*dIII (Eurobio). The agarose gels were analysed by densitometry (Gelworks 1D; UVP).

Qualitative analysis was performed by comparing the percentage appearance of each of the peaks of the control and exposed cells. After eliminating the background, quantitative differences were studied using three types of parameters: height, volume and percentage of the amplified band. Additionally, the individual data obtained on the height, volume and percentage of amplified bands were grouped together according to the following criteria: bands of high molecular weight (>800 bp), bands of intermediate molecular weight (between 600 and 800 bp) and bands of low molecular weight (<600 bp).

For statistical analysis all individual data on control and treated cells from

the different experiments were considered together. The data were analysed using Student's *t*-test and the Mann–Whitney *U*-test.

Results and conclusions

Primer concentration

In five of the 20 amplifications carried out with 5 pM of primer and without template, the appearance of 'ghost' bands of different molecular weights was noted as a result of polymerization of the primer with itself. When assays were performed with 2.5 and 4 pM, no artefacts were observed, so 4 pM primer was selected as the optimum for later assays.

Differences in the band pattern of control and treated cells

Qualitative analysis. The assays carried out with 5 and 20 ng of control genomic DNA resulted in six common amplification products, three considered as having high molecular weights (i.e. >800 bp), one with an intermediate molecular weight (700 bp) and two with low molecular weights (i.e. <600 bp). When the assay was carried out with 20 ng, apart from those described, three bands with a low appearance rate (erratic bands) were observed and therefore were not included in the pattern (Figure 1). Assays with 2.5 ng of genomic DNA generated the characteristic bands already defined, but they varied greatly in their intensity and frequency of appearance, so this concentration was discarded in later studies. In summary, in the assay conditions of 4 pM primer and 5 or 20 ng of DNA template, a constant pattern was established by those bands whose percentage of appearance was always >80% (Table I).

In genomic DNA extracts of the exposed cells, qualitative differences were observed in both concentrations when compared with the control cells, although these were greater when using 5 ng of genomic DNA. A drop in the appearance rate of bands was observed in treated cells at 5 ng for all the exposure periods. Band D was particularly affected, going from being present in 84% of the control assays to 50% at 4 h and 40% at 6 and 8 h of exposure, respectively. Five extra bands were also detected: band D1 appeared at all exposure periods, band F1 at 6 and 8 h, band A0 at 4 h and band C1 at 8 h. With 20 ng of template, a drop in the appearance rate of band D was also observed, decreasing by up to 74% after 6 and 8 h of treatment. No new bands were observed with this concentration (Table II).

Quantitative analysis. The control amplification products obtained for both DNA concentrations showed a constant fluorimetric profile and for the three quantitative parameters studied, band B presented the highest average values and band D the lowest. The values of these parameters behaved in the same way: Bands A, B, D and E coincide and differences observed in bands C and F are directly and inversely proportional to the genomic DNA concentration, respectively (Figure 2). In exposed cells, significant statistical differences ($P < 0.05$) versus the control were found using 5 ng of template for most fingerprint bands at all exposure periods as a function of the parameter analysed. The most significant differences ($P < 0.0001$) were observed at 8 h of exposure, when considering the percentage of the amplified band (Table III). These results contrast with those obtained when the concentration of genomic DNA used was 20 ng. No band showed significant overall differences in the parameters considered and, furthermore, the values obtained for the percentage of amplified bands coincide, for all exposure periods, with those of the control pattern (Table IV).

When each band is analysed individually, it is noted that the results for the control and exposed data are greatly dispersed. However, if data are grouped according to their molecular weights, this variability decreases and the differences between the control and treated cells are seen more clearly. When using this method with 5 ng of template, differences are noticeable at 6 and 8 h of treatment and the 5:2 ratio between the high and low molecular weight bands of the control was modified to a value of 1:1 (Table V). The differences were seen as a decrease in the amplification of the high molecular weight peaks in favour of the lower molecular weights. Conversely, at 4 h the differences were seen as a decrease in amplification of the low molecular weight peaks in favour of the high molecular weight peaks (bands C and F).

Statistically significant differences ($P < 0.01$) were obtained for all exposure periods in the low molecular weight band group (<600 bp) for all parameters, as well as in the high molecular weight band group (>800 bp), but only when the band percentage is considered. Although the parameters used for the quantitative valuation are all related, the height varies due to the electrophoretic process itself and the volume has a great dispersion of data. As the band percentage is a value relative to the total amplification of each reaction, we consider this latter as the most appropriate parameter for quantifying our results as it allows for a better comparison between gels. No differences were found with 20 ng of template even when the bands were grouped according to their molecular weights.

In conclusion, qualitative and quantitative differences were found in RTG-2 cells after exposure to 0.5 μg/ml MMC, but only when the template concentration was lowered to 5 ng of genomic DNA (Figure 3).

Discussion

Template concentration affects the electrophoretic band pattern and is the major source of non-reproducibility in RAPD fingerprint analysis, as previously described by other authors using different species (Muralidharan and Wakeland, 1993; Sakallah *et al*., 1995). In our case, 5 and 20 ng of genomic DNA from RTG-2 cells show variations in the intensity of amplification of two of the bands (C and F) and in the appearance of erratic bands at the higher concentration (Table I and Figure 2). The template concentration is also a key factor in showing differences in the band pattern between control and exposed cells. While some qualitative differences occur at both concentrations, such as a decrease in the frequency of the appearance of some bands, the appearance of new bands as well as quantitative alterations can be observed only when the template concentration is reduced (Figure 3).

Amongst the new bands observed in the exposed cells, band D1 (645 bp) was detected for all the exposure periods and the frequency of its appearance increased with the length of exposure. This leads one to think that these bands are the result of a MMC-directed action on specific points on the genomic DNA. This is in accordance with the specificity of MMC in CG-rich areas (Kumar *et al*., 1997; Palom *et al*., 1998; Warren *et al*., 1998).

Quantitative differences in control cells can be observed in the intensity of amplification by decreasing the amount of genomic DNA from 20 to 5 ng. This same effect, but much more noticeable, is found when comparing the control and exposed genomic DNA using 5 ng.

MMC is a well-known mutagenic and clastogenic agent;

base substitutions, sister chromatid exchanges, chromosomal aberrations and micronuclei, both *in vivo* and *in vitro*, are genetic effects that can be observed as a function of dose and exposure period (Pelt *et al*., 1991; Rudd *et al*., 1991; Salvadori *et al*., 1994). The dosage selected for this work was in accordance with previous studies of micronucleus induction in our laboratory on the same cell line (Llorente *et al*., 1998) and is also in the range for the positive control in *in vitro* genotoxicity assays (Ribas *et al*., 1998). Although MMC provokes alterations in the DNA of the cell population, with the concentration and exposure periods used in this work a certain proportion of DNA copies remain intact. In the case of 20 ng, the number of unaffected copies is sufficient that with the concentration of primer used, it finds sections where it can hybridize and therefore the characteristic band pattern is not altered. This is supported by the fact that only the scarcest bands (bands D and E, which represent 2.5 and 8% of the total amplified, respectively) are affected at the longer exposure periods (Table V). Nevertheless, when the amount of template is lowered to 5 ng, despite the fact that in relative terms the proportion of intact genomic DNA is the same, the number of original copies to be amplified is now much lower and insufficient, which increases the probability of the altered genomic DNA showing itself. The result is that a band pattern different to that of the control is obtained.

Smaller but significant quantitative differences between control and exposed cells for the shorter studied period can be observed as an opposite tendency when compared with longer exposure periods. In the RAPD reaction a high number of different sequences are co-amplified in the first cycles and the products from the most stable primer–template unions are the 'winners' of this process (Davin-Reglin *et al*., 1995). The observed tendency for 4 h could suggest that short exposures (4 h represents 1/12 of the population doubling time for this cell line) may lead to the production of mutations with changes in base pairs (Srikanth *et al*., 1994; Maccubbin *et al*., 1997) which can give the primer the opportunity to find new hybridization sites and obtain products of similar molecular weight but with different sequences (Reisenberg, 1996). Attention should also be paid to the qualitative analyses. After 4 h, a new high molecular weight band appears and the frequency of appearance of band C decreases by up to 90%, so that new and possibly unstable hybridizations may be suspected. Longer exposure periods lead to a decrease in the amplification of high molecular weight bands as a result of the clastogenic action of MMC (Pelt *et al*., 1991; Channarayappa and Ong, 1992).

Although RAPD assays are usually associated with inherent problems of the technique which prevent its routine application (Hadrys *et al*., 1993), if the conditions of the assay were optimized these inconveniences can be solved, i.e. appearance of 'ghost' bands, reproducibility, etc. (Theodorakis *et al*., 1998). By optimizing the RAPD conditions, and bearing in mind that RAPD are PCR products, they can be cloned and sequenced to obtain a wider knowledge of genotoxicant action mechanisms (Theodorakis *et al*., 1998).

The results of this work shows that the RAPD technique could be a promising tool in the *in vitro* detection of alterations in DNA produced by genotoxic agents, allowing us to see the mechanisms of action of the agent in greater detail and also permitting areas or 'hotspots' within the affected genomic DNA to be seen. Moreover, the practically unlimited number of informative primers provides a good overall coverage of genomic DNA (Ramser *et al*., 1996) so that the choice of a set of primers with different sequences will allow increased sensitivity of the assay in the detection of low frequency mutation events.

The purpose of this work was to study the capability of this technique to show genotoxic effects induced by chemicals in species where a long cell cycle, low ability of colony formation and small or high numbers of chromosomes (Babich and Borenfreund, 1991; Al-Sabty and Metcalfe, 1995) makes application of most mutagenicity assays impractical. Obviously, a large number and types of genotoxic agents must be tested to establish sensitivity limits before routine application of this assay.

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ANEXO 2.3

DNA FINGERPRINT COMPARISON OF RAINBOW TROUT AND RTG-2 CELL LINE USING RANDOM AMPLIFIED POLYMORPHIC DNA. (2001)

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DNA Fingerprint Comparison of Rainbow Trout and RTG-2 Cell Line Using Random Amplified Polymorphic DNA

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Abstract. The detection of genotoxic effects using *in vitro* cell systems can be extremely useful in risk assessment procedures. However, care should be taken in the extrapolation of *in vitro* results since, amongst other factors, established cell lines may deviate from the genetic characteristics of their species. In this work, the genetic similarities between the RTG-2 cell line and rainbow trout individuals (*Oncorhynchus mykiss*) from several fish farms have been studied by the RAPD technique. Results show a significant analogy in the band patterns obtained for both systems, up to 73% of the bands composing the fingerprint of the RTG-2 cell line were found in all the individuals analysed. The inter-population similarity index (Lynch, 1990), considering the RTG-2 cell line as a population, gives a value of 0.931 between both systems. The dendrogram constructed from all the individuals, considering the RTG-2 cell line as just another individual of a single population, showed that the genetic structure of the cell line was not different from those of the other individuals tested. The strong genetic similarity of both systems, together with the previously proven capability of the RAPD technique to detect genetic alterations caused *in vitro* by genotoxic agents, can be very useful in genetic ecotoxicological studies.

Keywords: RAPD; fingerprint DNA; rainbow trout; RTG-2; fish cell line; genotoxicity

Introduction

Genotoxic chemicals of anthropogenic origin released into the aquatic environment can lead to DNA structure alteration of exposed individuals. It has been demonstrated that the incidence of neoplasic processes rose in aquatic populations of contaminated areas (Sheridan, 1995; Pritchard et al., 1996). Both acute and chronic exposures to stressors can exert selective pressure upon organisms and the bioavailability of pollutants is strongly correlated with a decrease in survival and reproductive success within populations (Theodorakis and Shugart, 1997).

On the other hand, continued exposure to certain pollutants induces changes in the allelic frequency of the most sensitive genotypes amongst the exposed populations. Genotoxic compounds would, therefore, act as selecting agents, thus causing genetic bottlenecks with serious consequences for the population (Krane et al., 1998). The consequences of long-term diminished genetic diversity can be profound. Free-living popu-

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lations with high levels of genetic diversity use resources more broadly and efficiently than those with low levels of diversity (Bearmore et al., 1960; McDonald and Ayala, 1974).

Nowadays ecotoxicology has to face, among other problems, on one hand, how to extrapolate the laboratory test results to field situations and, on the other, how to extrapolate individual effects to the whole population. One of the main challenges in genetic ecotoxicology is finding methodologies to reliably value the effects that different degrees of pollution produce on biodiversity in the exposed natural populations. (Anderson, et al., 1994).

In the last few years, a large number of biomarkers at the molecular, cellular or individual levels have been developed (Walker, 1998) which are able to evaluate the effects caused by chronic and subchronic exposure to environmental pollutants. Toxicity- and biologically-based indexes have proven to be among the most sensitive and useful measures of environmental impacts. (McCarthy and Shugart. 1990). However, these field indexes are very laborious to obtain and do not allow one to establish a cause and effect relationship, except at a macromolecular level, that is, they do not allow one to elucidate the molecular mechanisms which iniciated originated the observed effects.

At present, molecular genetic markers have been developed, thus opening new perspectives in order to solve these problems.

One of them, the Random Amplified Polymorphic DNA (RAPD) technique developed by Williams et al., (1990) allows the detection of DNA polymorphisms by randomly amplifying multiple regions of the genome by PCR, using short arbitrary primers. The amplification products are resolved on agarose gels and the polymorphisms serve as genetic markers (Dinesh et al., 1993). Since the RAPD technique is powerful, simple, fast, sensitive and particularly suited to problems in which the genome is anonymous, it is now being applied to different fields such as molecular ecology, in order to determine taxonomic identities and detect the interspecific gene flow (Hadrys et al., 1992; Al-Zahim et al., 1997); food science to differentiate species (Koh et al., 1998; Szalai et al., 1997; Henry et al., 1997); and detection of mutations (Kubota et al., 1992; 1995; Savva, 1996; 1998; Conte et al., 1998; Atienzar et al., 1998; 1999; 2000; Becerril et al., 1999).

Predicting the effects upon populations of certain chemicals or complex mixtures prior to their release allows the establishment of safety levels in risk assessment protocols. In this sense, the *in vitro* methods which use cells of representative species offer valuable information in preliminary research and are irreplaceable from an economic point of view. The biggest problem of the *in vitro* methods is the extrapolation of results. Previous studies are necessary in order to demonstrate the suitability and reliability of the results thus establishing their degree of representativeness. To validate *in vitro* genotoxicity research, based on DNA fingerprint alterations, it is essential to compare both band patterns (*in vitro/in vivo*) and then establish their degree of similarity.

Fish species show a great variety in their chromosomic arrangement since, on one hand, they alter depending on the different environmental situations, giving as a result individuals with different morphology and chromosome number, and on the other, in stress situations they can lack standard karyotypes showing polymorphic differences within the same species (Al-Sabty, 1991).

The rainbow trout, a salmonoid fish, is among the most sensitive species because of its strict water quality requirements, and it is frequently used in aquatic toxicology due to its economic relevance and its representativeness. This species shows variations in its karyotype, finding diploid chromosome individuals (56–60 chromosomes), tetraploids (120 chromosomes) and even aneuploids (59, 61, 63 chromosomes) (Al-Sabty, 1991). Usually established cell lines lose many characteristics of their original cells, and even aneuploidy processes have been detected. The RTG-2 cell line, derived from the gonadal tissue of the rainbow trout (Wolf and Quimby, 1969) shows a chromosome number of 59 ± 2 (Hay et al., 1988).

Considering the genetic differences present in the RTG-2 cell line and the species it originated from, it is essential to know their RAPD band patterns and to establish the most representative primers that will allow one to obtain a constant and specific fingerprint for both systems. In previous research, the RAPD band pattern for this cell

line was obtained with different primer sets (Ferrero et al. 1998), and the capacity for detecting fingerprint alterations after cells were exposed to known mutagens was demonstrated (Becerril et al., 1999).

This work aims to establish the degree of similarity between the RAPD band patterns *in vitro/in vivo* with the set of primers previously established with RTG-2 and to determine the constant or polymorphic bands of both systems, selecting those primers giving rise to the most common bands. This way, the *in vitro* genotoxicity test results can be reliably extrapolated to *in vivo* conditions.

Materials and methods

In vitro material

The RTG-2 fish cell line (American Type Culture Collection, CCL 55), an established fibroblastic cell line derived from rainbow trout, (*Oncorhynchus mykiss*) was grown in Eagle Minimum Essential Medium with Earle's salts (EMEM), supplemented with 10% foetal serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 1.25 μg/ml fungizone and 2 mM L-glutamine, in PVC tissue culture flasks (Costar) at 20 $\pm$ 1°C in 5% CO_2/air atmosphere.

Cells were detached from the substratum with trypsin-EDTA (Flow, Scotland), collected in PBS pH 7.3 and then had their DNA extracted. Cells from different passages were used in the analysis.

In vivo material

Three groups (T0, T1 and T2) of rainbow trout (*O. mykiss*) individuals from of different fish farms and collected at different years were used in this work. The total number of fish was 33 (14, 7 and 12 individuals for T0, T1 and T2 respectively). It was not known if individuals collected from the same fish farm and at the same time came from the same hatching.

A blood volume of 1% of the body weight was drawn from the caudal vein, collected in heparinized haematocrit tubes and immediately centrifuged. The pellet was resuspended in citrate buffer pH 7.4 and aliquots of 100 μl were immediately frozen and stored at -70°C.

Each blood aliquot, once defrosted, was centrifuged and collected in PBS pH 7.3 and then had the DNA extracted.

DNA extraction and RAPDs reaction

The DNA was extracted by phenol-chloroform and its integrity was checked by electrophoresis in 0.8% agarose gels using λ Phage DNA native as molecular weight marker (Eurobio, France). The RAPD test was performed using 5 ng of DNA per reaction in a Perkin-Elmer model 2400 Thermalcycler (Becerril et al. 1999).

A total of 8 primers (Table 1) from the 32 previously tested *in vitro* (Ferrrero et al. 1998; Becerril et al. 1999) were used in the *in vivo/in vitro* comparisons. All primers were obtained from

Table 1. Characteristics of the primers used

Primer identification	Sequence	Total of amplified bands	Percentage of polymorphic bands yielded
AA-82	5′-GATCCATTGC-3′	9	55.6
AA-89	5′GGGCCTCTGAT-3′	15	53.3
ALU-2	5′-GACCCGCACC-3′	8	37.5
C-95	5′-CGGCCACTGT-3′	11	36.4
C-96	5′-AGCACTGTCA-3′	9	44.5
D-4	5′-CTGTAGCATC-3′	1	0
D-8	5′-CCAAGTCGACA-3′	7	28.6
tRNA-1	5′-AGTCCGGTGTGCTCTA-3′	13	38.5

Primers used in the *in vivo/in vitro* amplifications. The number of bands obtained were ranged from 1 to 15 per primer. All the primers used, except one, show polymorphic bands

the Biopolymers Department of the CNBCR, (I.S. Carlos III, Majadahonda, Madrid).

All DNA amplifications were carried out in duplicate and a negative control reaction, without template DNA, was included in the same gel. The amplification was repeated two or three times on different days.

Amplification products together a molecular weight marker (marker VI, Roche, Germany; or $\phi \times 174$-*Hae* III EUROBIO, France) were resolved electrophoretically on 2.1% agarose gels at 150 V for 4 hours and stained with ethidium bromide. The image was recorded by the Grab-it program (UVP, USA) and analysed by densitometry (Gelworks 1D, UVP, USA) in order to avoid operator subjectivity. Only repetitive bands (absent or present) in different gels taken on different days were considered for the analysis.

Data analysis

Each individual was coded with two digits: the first one referred to the population and the second one to the individual. The bands were coded following the system described by Theodorakis and Shugart (1997).

The presence or absence of each of the bands was determined by making a binary matrix (1 for band presence and 0 for absence) for every individual. This allowed one, on the one hand, to calculate the band frequency (as percentage of individuals within a population showing given band) and on the other, to compare intra and inter-population band patterns.

The analysis of the results was done in two ways: a) considering T0, T1, T2 and RTG-2 as different populations and b) considering all individuals, including the RTG-2 cell line, as members of the same population.

In order to establish the degrees of similarity, the coefficient of similarity among individuals (S_{sy}) was calculated from the band presence-absence matrix:

$$S_{sy} = n_{xy} | n_x + n_y$$

(Simple Matching Coefficient; Sokal and Michener, 1958) where n_{xy} is the number of bands (both present and absent) shared by the x and y individuals and n_x and n_y are the total number of bands scored for each individual.

The intra-population similarity index (S) was calculated as the average of all similarity coefficients among individuals of a population (S_{sy}, average).

The inter-population similarity index (S_{ij}) was calculated according to Lynch (1990) which introduces a similarity intra-population correction factor:

$$S_{ij} = 1 + S'_{ij} - 0.5(S_i + S_j).$$

where S_i and S_j are the values of S for population i and j, respectively. S'_{ij} is the average between randomly paired individuals from populations i and j. It is possible for the value of S_{ij} to exceed 1.

The intra and inter-population similarity index (S and S_{ij}) were calculated both separately for each primer and for all the primers as a whole.

Inter population similarity S_{ij} is inversely proportional to the genetic diversity (D) or dissimilarity index $D = 1 - S_{ij}$, whose range is between 0 (population completely homogeneous) and 1 (population completely heterogeneous).

D values were used to construct dendrograms, considering, as previously stated, a) T0, T1, T2 and RTG-2 as different populations and b) all individuals as members of a single population (in this case $D = 1 - S_{xy}$).

Dendrograms were constructed by two methods: the UPGMA method (Unweighted Pair-Group Method Analysis; Sneath and Sokal, 1973) and Njoin (Neighbor Joining; Saitou and Nei 1987) using the NTSYS-pc2 program (Numerical taxonomy and Multivariate Analysis system; Rohlf, 1990).

Results

In vitro / in vivo band patterns

With the primers set used in the *in vivo/in vitro* tests, a total of 73 reproducible fragments ranging from 2045 to 335 bp were obtained. The number

of bands per primer varied between 15 and 1 (primers AA-89 and D-4 respectively).

41 out of the 73 bands (more than 50% of the total) showed an appearance rate of 100% in both *in vivo* and *in vitro* assays. The rest were polymorphic in the *in vivo* amplifications. Some of these bands, 17 in particular, do not appear in the RTG-2 cell-line band pattern, whose constant fingerprint is made up by 56 bands. (Fig. 1).

The percentage of polymorphic bands was in general less than 50% of bands generated by each primer. Only the AA-82 and AA-89 primers presented a slightly higher frequency of polymorphism (55.6% and 53.3% respectively) (Table 1).

As shown in Table 2, the frequency of polymorphic bands appearance in the different populations is related with the presence or absence of those bands in RTG-2. Therefore, bands present on RTG-2 appear with a high frequency (more than 50% of the cases) in the populations and those absent appear with an inferior rate (less than 50%). There are, however, some exceptions: two of the bands show clear *in vivo* and *in vitro* differences. The tRNA-1_{1494} band found in RTG-2 appeared only in 14% of the individuals of the T0 population. On the contrary, the C-95_{974} band, absent in RTG-2, shows an amplification percentage of over 75% in all the populations.

Population analysis

Considering all the bands, the *in vivo* intra-population similarity index (S) ranged from 0.848 to 0.861 except for RTG-2, that due to a constant DNA fingerprint, shows a value of 1.

Inter-population similarity indexes (S_{ij}) were higher than the previous ones. They varied from 0.91 to 0.942 between RTG-2 and the *in vivo* populations, obtaining an average *in vitro/in vivo* inter-population index of 0.931 (Table 3).

Higher values (0.981 to 0.999) were obtained among the *in vivo* populations, being practically 1 between T0/T1.

The lowest inter-population values are obtained between the T2/RTG-2 populations with AA-82, C-95 and tRNA-1 primers.

Dendrograms using UPGMA and Njoin methods, considering T0, T1, T2 and RTG-2 as different populations, are shown in Figure 2. The grouping of the *in vivo* populations varied depending on the method used. However, in both cases the RTG-2 cell line appears to be clearly related to the *in vivo* populations.

When considering all individuals, as belonging to a single population, including the RTG-2 cell line, the resulting dendrogram (Fig. 3) shows two branches indistinctly formed by individuals

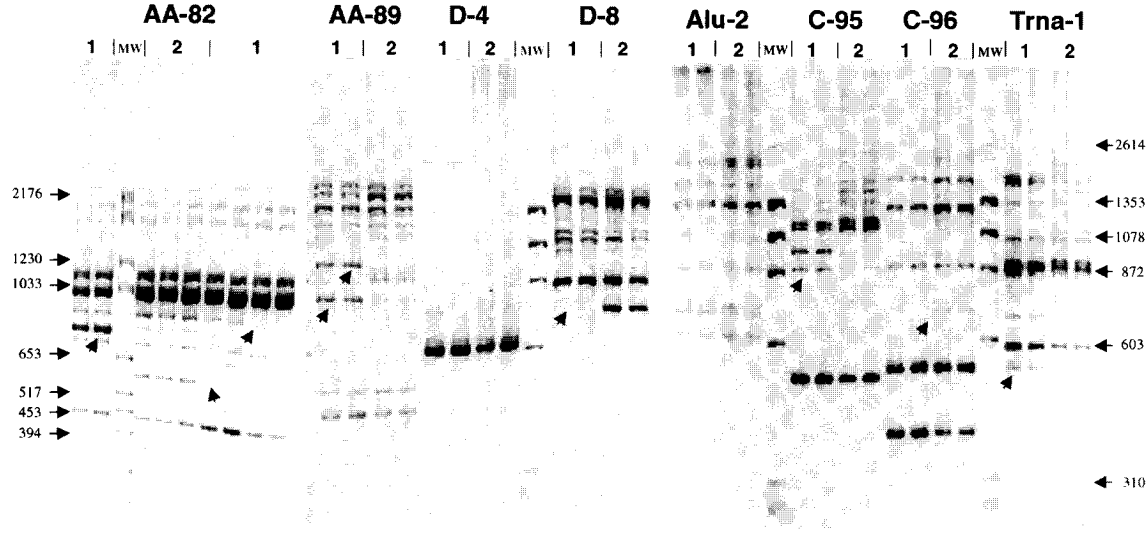

Figure 1. *In vivo* (1) and *in vitro* (2) DNA fingerprints obtained with the primers used in this work. Arrows show presence or absence of different polymorphic bands. MW: molecular weight marker.

Table 2. Frequency of appearance of *in vivo* polymorphic bands shared by RTG-2 cell line

Present in RTG-2				Absent in RTG-2			
	Percentage per population				Percentage per population		
Bands	T0 n = 14	T1 7	T2 12	Bands	T0 n = 14	T1 7	T2 12
AA-82_{570}	64	86	8	AA-82_{930}	29	14	0
AA-82_{430}	86	100	100	AA-82_{725}	43	30	39
				AA-82_{640}	21	43	38
AA-89_{867}	100	86	100	AA-89_{1459}	21	43	8
AA-89_{698}	100	86	100	AA-89_{1029}	8	20	8
AA-89_{463}	58	40	48	AA-89_{549}	17	20	48
AA-89_{450}	43	59	42	AA-89_{453}	26	37	46
ALU-2_{1085}	86	100	92	ALU-2_{1130}	14	0	17
ALU-2_{590}	57	100	75				
C-95_{1151}	31	80	83	C-95_{974}	78	100	75
C-95_{468}	100	100	83	C-95_{634}	22	20	33
				C-95_{433}	21	14	50
C-96_{380}	86	100	100	C-96_{1646}	14	14	8
C-96_{642}	71	86	67	C-96_{598}	36	26	33
D-8_{686}	29	58	50	D-8_{638}	0	14	0
tRNA-1_{538}	64	100	25	tRNA-1_{964}	14	29	42
tRNA-1_{1494}	14	0	0	tRNA-1_{497}	21	29	17
				tRNA-1_{376}	0	0	8

Absence/presence frequency of polymorphic bands in the fish populations considered (T0, T1 and T2) in relation to the RTG-2 cell line. Bands present *in vitro* appear with a high frequency (more than 50% of the cases) while the absence of bands shows an inferior concordance *in vivo/in vitro*. Two bands show clear *in vivo/in vitro* differences, tRNA-1_{1494} is practically absent *in vivo* and C-95_{974}, absent in vitro, shows a percentage of amplification over 75% *in vivo*. (n, number of individuals per population).
Each band is indicated by the primer which was used to amplify it, followed by its molecular length in subscript.

Table 3. Inter and intra-population similarity indexes

PRIMERS	RTG-2/T0	RTG-2/T1	RTG-2/T2	T0/T1	T0/T2	T1/T2	T0	T1	T2	RTG-2
AA-82	0.9573	0.9683	0.8249	1.0071	0.9458	0.9413	0.7680	0.8095	0.8687	1
AA-89	0.9319	0.8857	0.9152	1.0072	1.0058	1.0072	0.8315	0.7714	0.7714	1
D-8	0.9294	0.9320	0.9675	1.0071	0.9989	1.0015	0.9213	0.8912	0.9082	1
C-96	0.9470	0.9683	0.9676	1.0068	1.0099	1.0126	0.8361	0.8730	0.8611	1
C-95	0.9381	0.9567	0.8636	0.9857	0.9338	0.9545	0.8511	0.8788	0.8485	1
Alu-2	0.9463	0.9683	0.9646	1.0098	1.0157	1.0030	0.8376	0.8732	0.8670	1
tRNA-1	0.9324	0.9158	0.8683	0.9957	0.9884	0.9635	0.8935	0.9267	0.8916	1
Mean	0.9403	0.9421	0.9102	0.9996	0.9855	0.9834	0.8484	0.8605	0.8595	1
SD	0.010	0.032	0.059	0.009	0.033	0.029	0.049	0.052	0.044	

Inter and intra-population similarity indexes for each primer separately and considering the data primers as a whole.

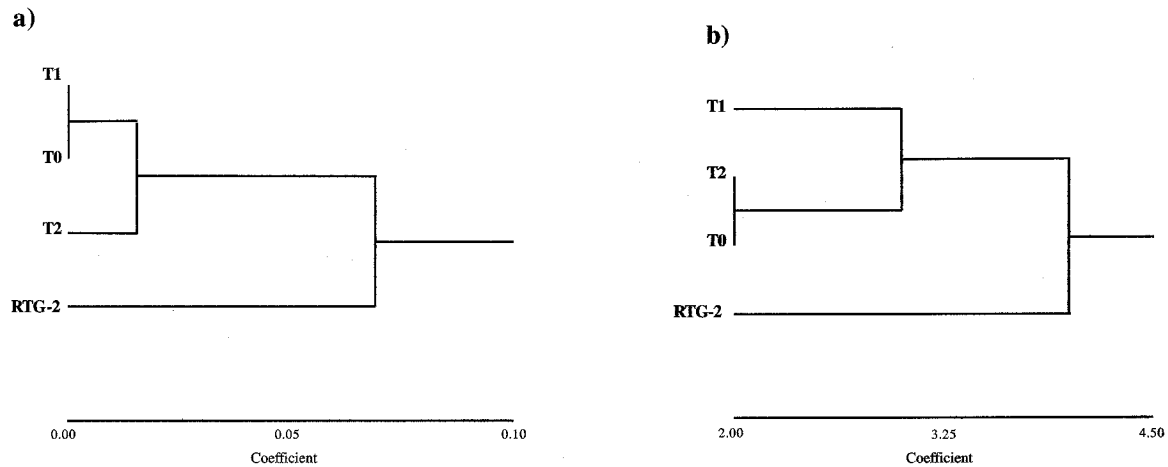

Figure 2. Dendrograms constructed with the *in vivo* (T1, T2 and T3) and *in vitro* (RTG-2 cell line) populations using: a) UPGMA (unweighted pair-group method average) method and b) Njoin (Neighbour-joining) method.

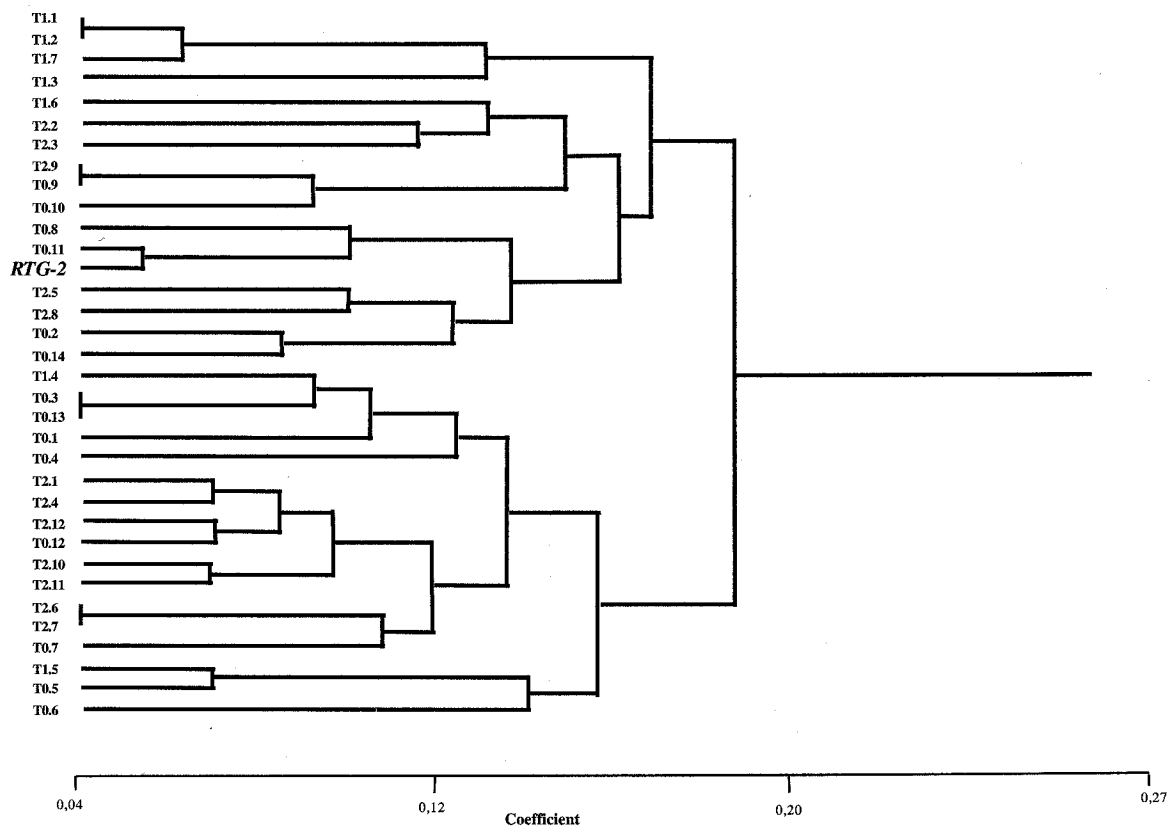

Figure 3. UPGMA dendrogram considering all individuals (including RTG-2) as a single population.

from all populations (T0, T1 and T2). The RTG-2 cell line is located in one of the branches and shows a close genetic relation to the individuals analyzed, behaving like any other individual of the population.

Discussion

The use of *in vitro* tests carried out with cell cultures for genotoxicity assays is widely accepted in the classification protocols for chemicals (OCDE guidelines n° 473, 474, 476, 479, 482). The application of in vitro tests to ecotoxicology both for pure chemicals and complex mixtures could be a useful tool in the first steps of risk assessment studies. However, when using *in vitro* tests for genotoxicity assays detecting DNA fingerprint alterations, it should be noted that, in general terms, established cell lines may change their genetic complement and differ from the original cells.

With the knowledge of the *in vivo/in vitro* similarity rate obtained by the detection of common and/or exclusive bands for either systems, extrapolation of results could be more precisely achieved.

The *in vivo* and *in vitro* band patterns obtained in this work show a significant concordance, as 73% of the bands making up the fingerprint are present in all the rainbow trout individuals of the analysed populations (only 15 bands of them were polymorphics (Table 2)).

On the other hand, it should be noted that DNA fingerprints obtained from rainbow trout individuals show two remarkable differences when compared to the DNA fingerprint obtained with RTG-2 cell line:

1. A higher number of bands (73 versus 56 for RTG-2). The lower band number in RTG-2 is probably directly related to the homozygous character of the cell line. This has been suggested by other authors comparing completely homozygous fish with those which possess at least one heterozygous locus (Theodorakis, et al 1997).
2. Approximately 50% of these bands are polymorphic demonstrating, the genetic variability of the species. This is a direct consequence of the RAPD technique, which in theory, permits one to analyse an unlimited number of loci (Lynch and Milligan, 1994).

The percentage of polymorphic bands detected varies depending on the primer in use (AA-82 and AA-89 exceed 50% of the total bands amplified) (Table 1), but probably these regions of the genome present a higher variability and more susceptibility to alteration by external agents than those more conserved regions, that would generate constant bands in both systems.

In general, with the exception of C-95 and tRNA1 primers, a direct relation can be established between the percentage of *in vivo* appearance and the presence or absence of those bands in the RTG-2 cell line.

Although the number of individuals analysed is not very high, the results obtained show that primers C-95 and tRNA-1 have bands that could be considered specific to one system or the other. Therefore $tRNA\text{-}1_{1494,}$ present in the RTG-2 cell line, was only detected in one individual belonging to one of the studied populations (T0) and $C\text{-}95_{974,}$ absent from the DNA fingerprint of the RTG-2 cell line, shows a high percentage of appearance in all the *in vivo* populations (Table 2). Taking these results into account, primers tRNA-1 and C-95 will not be included in our subsequent genotoxicity assays.

The individuals used in this work were collected from two different farms and in very distant periods (up to three years), so it was assumed that these lots belonged to different populations. However, population studies show the lack of differing characteristics in the established populations, presenting very high inter-population similarity indexes (0.999–0.983) and the much lower intra-population indexes (0.860–0.848), show that individuals from every population are scarcely homogeneous (Table 3). Working with populations belonging to the same species of Barbus in the Iberian Peninsula, Calleja, (1998) finds inter-population similarity indexes between 0.940 and 0.969.

There is no agreement in the genetic distance among the *in vivo* populations showed in the dendrograms using different methods (UPGMA and Njoin) (Fig. 2). Furthermore, the dendrogram constructed with all individuals shows an hetero-

geneous distribution of the individuals belonging to three populations and does not allow the establishment of any other kind of grouping (Fig. 3). All the above leads us to think that T0, T1 and T2 are not really independent populations but members of the same population that show a high degree of genetic variability.

The original fish farms are located in a nearby area, they do not work in closed cycle and probably their reproduction techniques foster continuous gene flow.

Population studies comparing the RTG-2 cell line to rainbow trout individuals show that this line has maintained a close genetic relation with the *in vivo* system it derives from, and with the selected primers both, rainbow trout and RTG-2 cell line, can be considered as two individuals belonging to a single population. (Fig. 3).

Although the RAPD technique is relatively new, and there is a limited information about reference values, the inter-population similarity index *in vivo/in vitro* obtained by us (0.931) is considerably higher than the one Bardakci and Skibinski (1994) found in Tilapia, where populations with a similarity index of 0.89 were considered as belonging to the same species. Likewise, in American seabass populations a lower value (0.884) was found (Bielawski and Pumo, 1997).

Other studies on inter-population similarity indexes for species belonging to the same genus are available in the literature and they show average values close to 0.758 (Calleja, 1998; Bardakci and Skibinski, 1994).

Bearing in mind the great genetic similarity showed by both *in vivo* and *in vitro* systems, alterations in the RTG-2 cell line produced by environmental genotoxic agents could also occur in the *in vivo* populations altering the genomic arrangement of the trout populations.

For genotoxicity studies, the RTG-2 cell line, as opposed to *in vivo* tests, presents the advantage that it can be considered as a population formed by a high number of homozygous individuals. After exposure to genotoxic substances, alterations in the band pattern will be due to their interaction with the DNA in a given number of cells.

The great simplicity of this system, together with its high genetic similarity as opposed to *in vivo* systems, makes it suitable for genetic ecotoxicity assays, not only to assess the effects of exposures to genotoxic agents (Becerril et al., 1999), but also to study the action mechanisms of those substances.

Furthermore, the presence of 'hotspots' in the genome, more susceptible to alterations by genotoxic agents (Warren et al., 1998.) makes a good concordance between the *in vivo* and *in vitro* genomic DNA of great interest for assessing the action mechanisms of genotoxic agents. All the above makes the use of cell cultures a real alternative method to *in vivo* fish bioassay.

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ANEXO 2.4

DETECTION BY RAPD OF GENETIC ALTERATIONS IN VITRO. AMPLIFICATION AND CONSERVATION CONDITIONS OF DNA EXTRACTS. (2002)

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Detection by RAPD of genetic alterations in vitro. Amplification and conservation conditions of DNA extracts.

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ABSTRACT

The technique of random amplified polymorphic DNA (RAPD) permits the study of genetic ecotoxicology without the problem posed by the high number of chromosomes generally presented by fish species for traditional methods. Additionally, the use of *in vitro* systems allows us to increase the number of samples in environmental risk assessment studies.

Good standardization of the parameters involved in the RAPD reaction, such as primer concentration, the DNA template used and its integrity is fundamental for obtaining reliable and repetitive results. This is specially important when the differences in the DNA fingerprints between control and exposed cells to genotoxic agents are interpreted as toxic-dependent alterations. The use of more than one primer increases sensitivity in the detection of such differences, provided the amplification is carried out in optimum conditions. In this report we show how the conditions established for certain primers in previous studies can be acceptable for others, independently of the complexity of band patterns generated.

Furthermore, the integrity of the DNA is shown to be stable for several months- in the genomic extracts stored at 4ºC, which to a large extent facilitates the application of this methodology.

Key word: RAPD, Genotoxicity, DNA alteration, MMC, DNA stability, RTG-2 fish cell line.

INTRODUCTION.

The increasingly frequent application of *in vitro* systems in toxicology is demonstrating the usefulness of alternative systems in the first stages of risk assessment of chemicals.

In environmental risk assessment procedures, and specifically in the evaluation of environmental pollutants, *in vitro* models are particularly useful because their low cost permits to process a high number of samples and, in contrast to the *in vivo* studies, the environmental conditions of the assays are easily controllable.

The established fish cell line RTG-2 derived from gonadal tissue of rainbow trout (Oncorrynchus mikyss) (1) has been widely used in cytoxicity and genotoxicity studies, showing a good correlation with *in vivo* bioassays (2, 3). Its use, together with molecular techniques, allows to perform genetic ecotoxicology studies without the problem the high number of chromosomes, characteristic of this species, has presented for traditional genotoxicity tests.

The Random Amplified Polymorphic DNA (RAPD) technique developed by Williams et al., (4) is a modification of the PCR technique that uses short sequence primers and low annealing temperature. Under these conditions, the primer links itself to various areas of the genome generating multiple fragments that, once developed using electrophoresis in agarose gels, form a template of bands specific for the genomic DNA used.

In the past few years this methodology has been applied to taxonomic studies and identification of species (5-8). In genetic ecotoxicology it has shown to be efficient for the detection of mutations (9-17), and for the detection of

modifications in the genetic diversity of the exposed populations (18, 19).

This technique has been associated with lack of homogeneity in the results for a number of reasons. Among them, variations in temperature profiles, particularly the annealing temperature (20-22), different thermostable DNA polymerases, salt concentrations (23) etc., can lead to a lack of reproducibility.

Aside from these factors, the primer and template concentrations are critical because they affect the products quantitatively (24, 25), and must be established and adjusted before initiating any study. When the protocols have been established very carefully, the results are sensitive and repetitive. (12).

Good standardization of each one of the parameters involved in the reaction is especially important when this technique is used for the detection of genotoxic alterations. In these studies small variations in the band pattern such as modification in intensity, loss and/or gain of bands are interpreted as alterations undergone by the DNA after being exposed to genotoxic agents.

In previous studies, the concentrations of genome and primer were established for the detection of changes in the DNA of RTG-2 cells exposed to Mitomycin C (MMC), using a primer that generated multiple fragments, between 1075 and 430 bp (12).

Since the best approach for this type of researches is the use of a battery of primers (26), in this study selected conditions of amplifications are evaluated to establish whether they can be generalized for any of the primers selected.

Furthermore, and independently of the standardized parameters, obviously the integrity of the DNA template is fundamental to the reproducibility of the results. The integrity of the DNA template depends on the

extraction method, the time and the storage conditions. Black (27) found changes in RAPD patterns due to template DNA degradation during freezing and thawing on consecutive days. Due to this, the DNA degradation occurring in the genomic extracts is evaluated additionally in this study over a period of almost two years.

For this:

a) The integrity of the DNA of 18 genome extracts stored at 4ºC over a period of almost two years was monitored .

b) The genomic DNA originated from RTG-2 control cells and exposed to 1 μg/ml of MMC for 4 hours was amplified using a primer whose band pattern is made up of a single amplification product.

MATERIAL AND METHODS

Culture cells

RTG-2 cells (28) from an established fibroblastic cell line obtained from rainbow trout (*Oncorhynchus mykiss*) were grown in Minimum Essential Medium Eagle with Earle´s salts (EMEM), supplemented with 10% fetal serum, 100 U/ml penicillin, 100 g/streptomycin, 1.25 μg/ml fungizone and L-glutamine 2mM, in PVC tissue culture flasks (Costar, Corning, NY) and incubated at 20 ± 1ºC in 5% CO_2/air atmosphere.

Exponential growing cells were exposed to 1 μg/ml MMC (Sigma, St Louis, MO) for 4 h. After this time the medium was withdrawn and after washing with pH 7.3 Phosphate Buffered Saline Solution (PBS), fresh medium

was added and the cells were left to grow for a further 72 h (approximately one and a half cell cycles).

Two 75 cm^2 flasks were used for each of the exposure times and also for the control. The experiment was repeated twice on different days.

ADN extraction and RAPD amplification.

Cells were dissociated with trypsin-EDTA (Flow Laboratories, Riskmansworth, UK), collected in BPS pH 7.3 and centrifuged at 3000 rpm for 10 min.

The DNA was extracted with phenol and the concentration and purity of DNA were calculated, with an additional reading at 320 nm, (Lamda 2, Perkin-Elmer, Branchburg, NY) (26). Its integrity was checked by electrophoresis in 0.8% agarose gels using λ Phage DNA native as molecular weight marker (Eurobio, Paris, France).

The genome extracts were aliquoted in Eppendorf tubes and stored at 4ºC for a total of twenty one months.

The amplifications were carried out in a volume of 25 µL, containing a buffer solution of Mg Cl_2, 2 U Stoffel fragments (Perkin-Elmer, Branchburg, NY) and 0,2 mM of each of the dNTP (Pharmacia, Barcelona, Spain). The DNA template and primers were added in different concentrations.

The RAPD protocol used consisted of an initial denaturing step of 3 min. at 92ºC, followed by 45 cycles at 92ºC for 25 s (denaturation), 36ºC for 75 s (annealing), and 72º C for 6 min. (extension). Five additional cycles were programmed at 92 ºC for 20 s, 36 ºC for 75 s, and 72 ºC for 7 min. Cycling

was concluded with a final extension at 72 ºC for 7 min. The thermal cycler used was a Perkin-Elmer, mod. 2400.

The primer used was D-4 (5´-CTGTAGCATC-3´) (supplied by the Biopolymers Department of the CNBCR, I.S. Carlos III, Majadahonda, Madrid, Spain). This primer was selected because it generates only one amplification product as opposed to the previously studied primer, which generates multiple products (12).

All DNA amplifications were carried out in duplicate and were repeated two or four times on different days.

Amplification products (22 μL), together with a molecular weight marker (Marker VI, Roche, Germany), were resolved electrophoretically on 2.1% agarose gels at 150 V for 4 hours and stained with ethidium bromide. The image was recorded by the Grab-it program (UVP, USA) and the band intensity was analyzed by densitometry (Gelworks 1D, UVP, USA).

Checking of optimum concentrations

Firstly, the cell control fingerprint was checked at different concentration levels: 20, 15, 10, and 5 ng DNA template and 6, 5, 4 and 2 pM primer.

To check the validity of this primer in the optimum conditions previously selected, (5 ng of DNA template and 4 pM of primer) (12) genomic DNA from control and exposed cells (20, 10, 5 y 2 ng/μL) was amplified using 4 pM of primer. To check the results and avoid possible artifacts, the control and exposed genomic DNA were amplified in duplicate and run in the same gel.

The possible qualitative and quantitative differences between the DNA from control and exposed cells in the agarose gels were analyzed by

densitometry (Gelworks 1D; UVP). The quantitative analysis was carried out by comparing peak height between control and exposed cells.

For statistical analysis all individual data on control and treated cells from the different experiments were considered together. The data were analyzed using the Mann-Whitney *U*-test.

Genomic DNA extract stability stored at 4ºC.

A total of 18 genomic DNA extracts (42-18 μg/μL) were stored at 4ºC for 21 months. To monitor the stability of these extracts, the concentration and integrity of the genomic DNA were checked using the previously described method. These tests were carried out at regular intervals for each of the stored extracts (0, 3, 6, 9, 15 y 21 months).

RESULTS

Optimum DNA template concentration.

The D-4 primer shows a constant band at 578 bp in all the genome and primer conditions tested. Furthermore, when primer concentrations of 6 and 5 pM are used in the reaction, a variable number of erratic fragments of different molecular weights appear (Fig.1), independently of genomic DNA concentrations tested (20 – 5 ng).

When the DNA template concentrations used in this study are amplified with 4 pM of primer, the erratic fragments, though present in some amplifications, are of such low intensity that they can effectively be considered as non-existent. When the primer concentration is decreased to 2 pM the erratic bands disappear (Fig. 1).

Figure 2, shows the quantitative differences in amplification intensity of the 578 bp fragment between control and exposed cells when genome concentrations of 10 ng or less are used.

These differences, quantified using peak height values, were statistically significant for 10 and 5 ng of genome template ($p<0.05$ y $p<0.001$ respectively) (Table1). At lower concentrations (2 ng) quantitative analysis was not possible due to frequent amplification losses of the DNA from the exposed cells (Fig.2).

Stability of genomic DNA at 4ºC.

The spectophotometric studies carried out show DNA concentrations similar to initial concentration in all the extracts at all times (Fig 3).

The electrophoretic studies showed that genomic DNA was intact in 100% of the extracts during the first 9 months of storage. After this period, only 45.5 and 28.3% of the extracts (at 15 and 21 months respectively) maintained their integrity (Fig.4).

Accordingly, the DNA extracted in the manner described and stored at 4ºC can be used as a template in the RAPD amplifications for at least nine months after its extraction.

DISCUSSION.

Detection of genotoxic effect using these techniques involves the comparison of band patterns generated from control (unexposed) and treated (exposed) DNA from a set of previously selected primers.

Differences in profiles such as variations in the band number or changes in amplification intensity of some of the bands show alterations in the DNA sequence of exposed cells.

Such changes are attributed to a loss or gain of a priming site owing to specific mutations at one or both primer annealing sites on the DNA strand and/or large deletions and/or homologous recombination in a sufficient number of cells. The changes in amplified band fluorescence can be due, aside from the changes already described, to the presence of bulky adducts that potentially block the PCR enzyme (10, 24).

Given the existence of a variety of genotoxic contaminants with different mechanisms of action on the DNA, the possibility of showing this variety of damage will depend on the possibility of amplifying different regions of the genomic DNA of the exposed cells.

It is to be expected that the profiles obtained from a wide set of primers increase the sensitivity in the detection of differences between control and exposed DNA. However, although a good amplification can be obtained in a wide range of DNA and primer concentrations, each oligonucleotide provides an optimum value depending on the number of bands obtained (26), and the affinity of the DNA template-primer in specific work conditions (23). It is important, thus, to verify the optimum amplification characteristics of the primers used.

On the other hand, not all optimum primer and genome concentrations show differences in control and exposed amplification profiles. In a cellular population exposed to genotoxic agents a certain proportion of DNA copies can be remain intact, and if the number of unaffected copies present in the

DNA template is sufficient, the primer finds sections where it can hybridize and the characteristic band pattern is therefore not altered.

From a pragmatic point of view, if we can establish similar conditions for a set of primers, the assays could be obviously simplified.

Previous studies have shown that lower genome and primer concentrations (2 ng / 2 pM) for obtaining the fingerprint, are optimal for this primer (26), but, as we have demonstrated in this study, these low concentrations are responsible for a varying amplification loss of the exposed genomes, making quantitative dose (concentration) / response (effect) studies more difficult.

If we compare the 578 bp band height after amplification with 4 pM of primer and 20 - 2 ng of genomic DNA of control and exposed cells to MMC, only the amplifications carried out with 10 and 5 ng of genome show significant statistical differences ($p<0.05$ y $p<0.001$ respectively) (Table 1). At concentrations of 2 ng, genomic DNA obtained from exposed cells did not always amplify, indicating that the number of damaged copies at this concentration were not sufficient to visualize the product generated. (Fig.2)

This shows that the primer and DNA template (4 pM and 5 ng respectively) conditions chosen are adequate for detecting quantitative differences between control and exposed genomic DNA.

These conditions, which are coincident with those already established for the first primer (12), suggest its suitability, independently of the number of fragments that are generated.

The *in vitro* studies, as mentioned before, have the advantage of simultaneously evaluating a large number of samples. On some occasions this

involves extracting the DNA from a large number of samples simultaneously, to be subsequently processed.

The process of freezing and thawing can negatively affect the integrity of the genome (27), not permitting results with sufficient precision and reliability. This fact is often not detected by the operator. Furthermore, in many cases the DNA content present in each aliquot of the extract is low and does not permit to carry out of electrophoresis to check the integrity of the DNA each time it is used.

In our work conditions, 100% of the extracts stored at 4ºC maintain their integrity for nine months. Beyond this time, although DNA concentration in the extract remains practically constant (Fig.3), the integrity decreases and should not, therefore, be used as a template in the RAPD reaction.

The methodological advance offered by the RAPD technique in the detection of DNA alterations after exposure to genotoxic agents depends to a large extent on an effective standardization of all the parameters involved in the reaction. The possibility of storing genomic extracts at 4ºC, and the use of different primers with the same protocol considerably facilitates the application of this methodology.

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TABLE 1. Average data on height with 20, 15, 10 and 5 ng of genomic DNA for control and exposed cells (C and E respectively) for the band making up the fingerprint of D-4 primer.

ng DNA	20		15		10		5	
Exposure	C	E	C	E	C	E	C	E
Height ± SD	163 ± 14.1	154 ± 26.7	153 ± 22.5	143 ± 24.9	155 ± 26,3	102 ± 11,8[a]	147 ± 12.8	66 ± 18,5 [c]

Values are mean ± SD of 8 amplifications. Significant differences versus control. [a]$P < 0.05$, [b]$P < 0.001$, [c]$P < 0.0001$ by Mann-Whinney test.

FIGURES LEGENDS

Fig. 1. Fingerprints generated from DNA obtained from control cells using different concentrations of genome and primer. Using 6 and 5 pM of primer the amplifications show erratic bands of different molecular weights. (mw: molecular weigh marker)

Fig. 2. Comparison of the amplification intensity at different genomic DNA concentrations from control and exposed cells.

a) Differences in the amplification intensity are observed upwards of 10 ng of genomic DNA, (E exposed, C control), there with no amplification at lower concentrations tested.

   (1, 2, 3, 4 and 5 amplifications using 20, 15, 10, 5 and 2 ng of genomic DNA respectively).

b) Electrophoretic profile of control and exposed cells with 10 and 5 ng genomic DNA.

Fig. 3. Variations in the DNA concentration in the extracts and times studied.

Fig. 4. Checking Gel of genomic DNA integrity. Lanes **a** shows the presence of a high molecular weight band which indicates the good state of conservation of the genomic DNA, 0 month (a_1) and 9 months (a_2) after extraction. However, the smear showed by lanes **b** is a consequence of the disintegration of the DNA present in the extracts, 15 months (b_1) and 21 months (b_2) after extraction. (mw: molecular weigh marker)

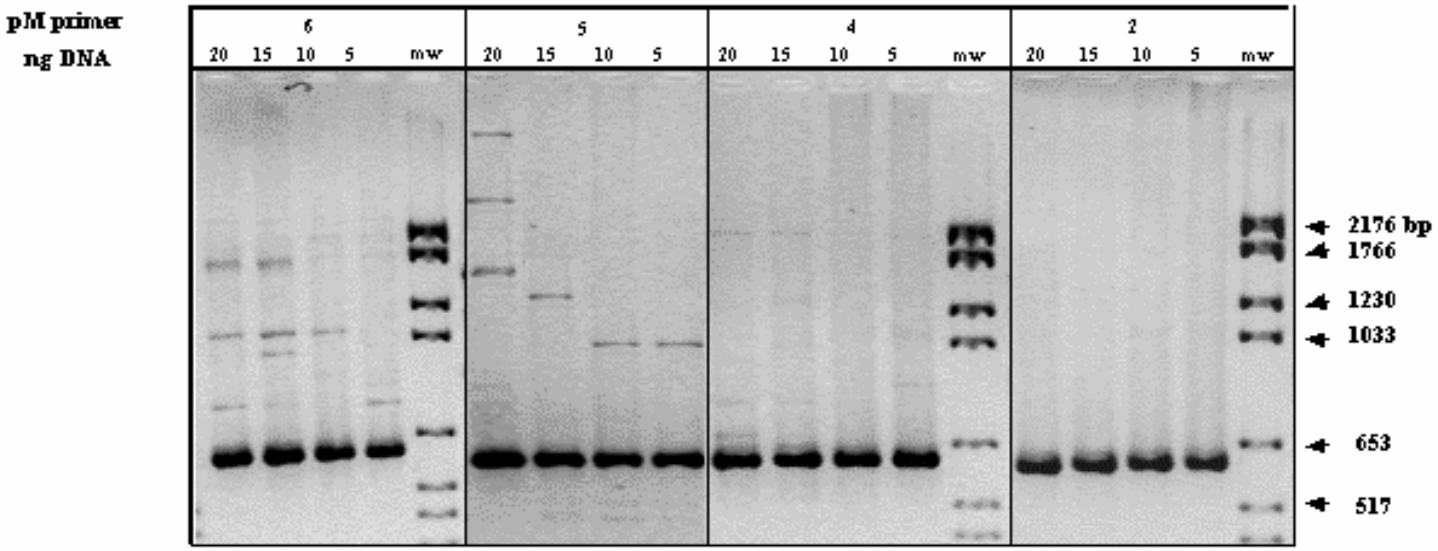


Figure 1. Fingerprints generated from DNA obtained from control cells using different concentrations of genome and primer.

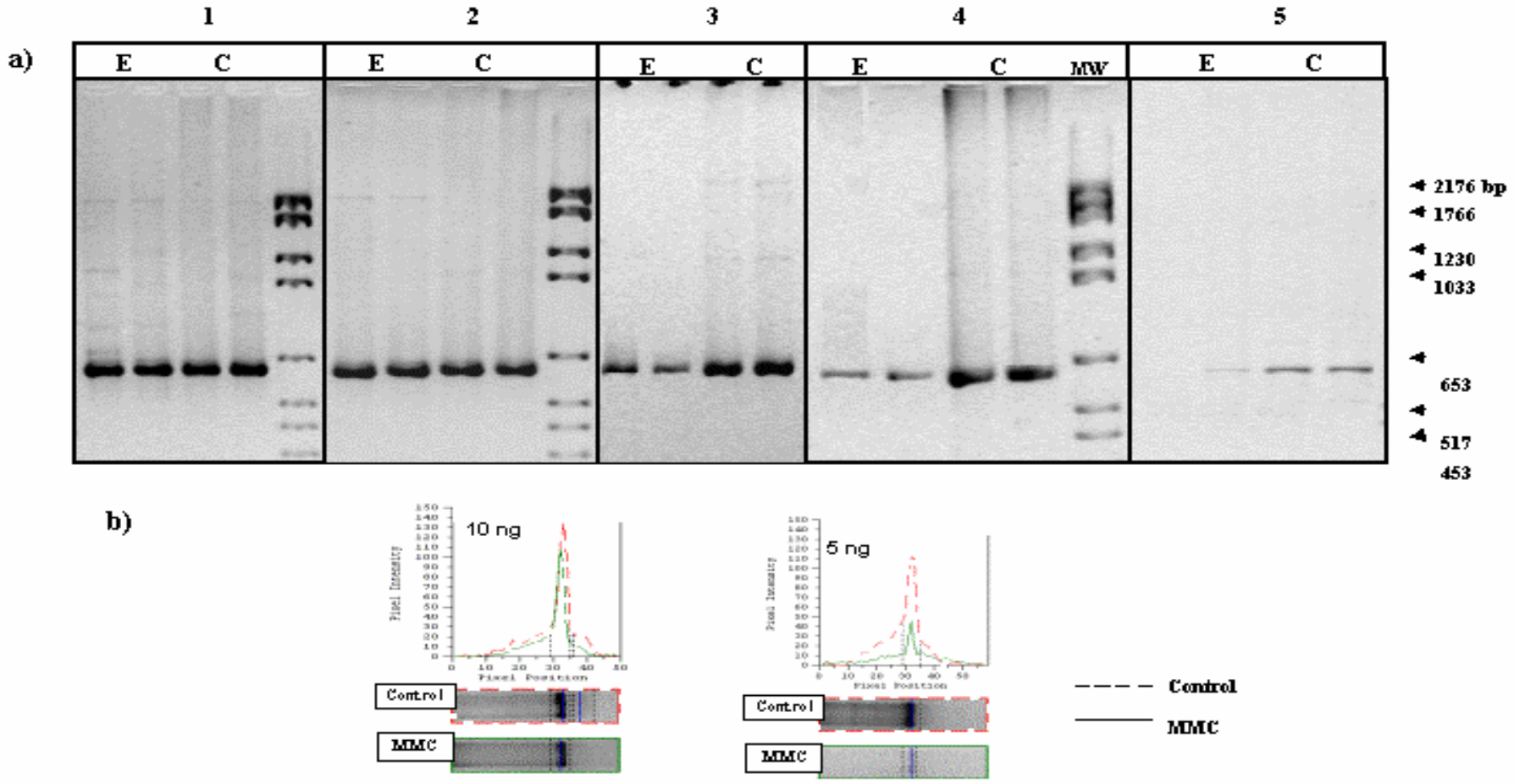


Figure 2. Comparison of the amplification intensity at different genomic DNA concentrations from control and exposed cells.

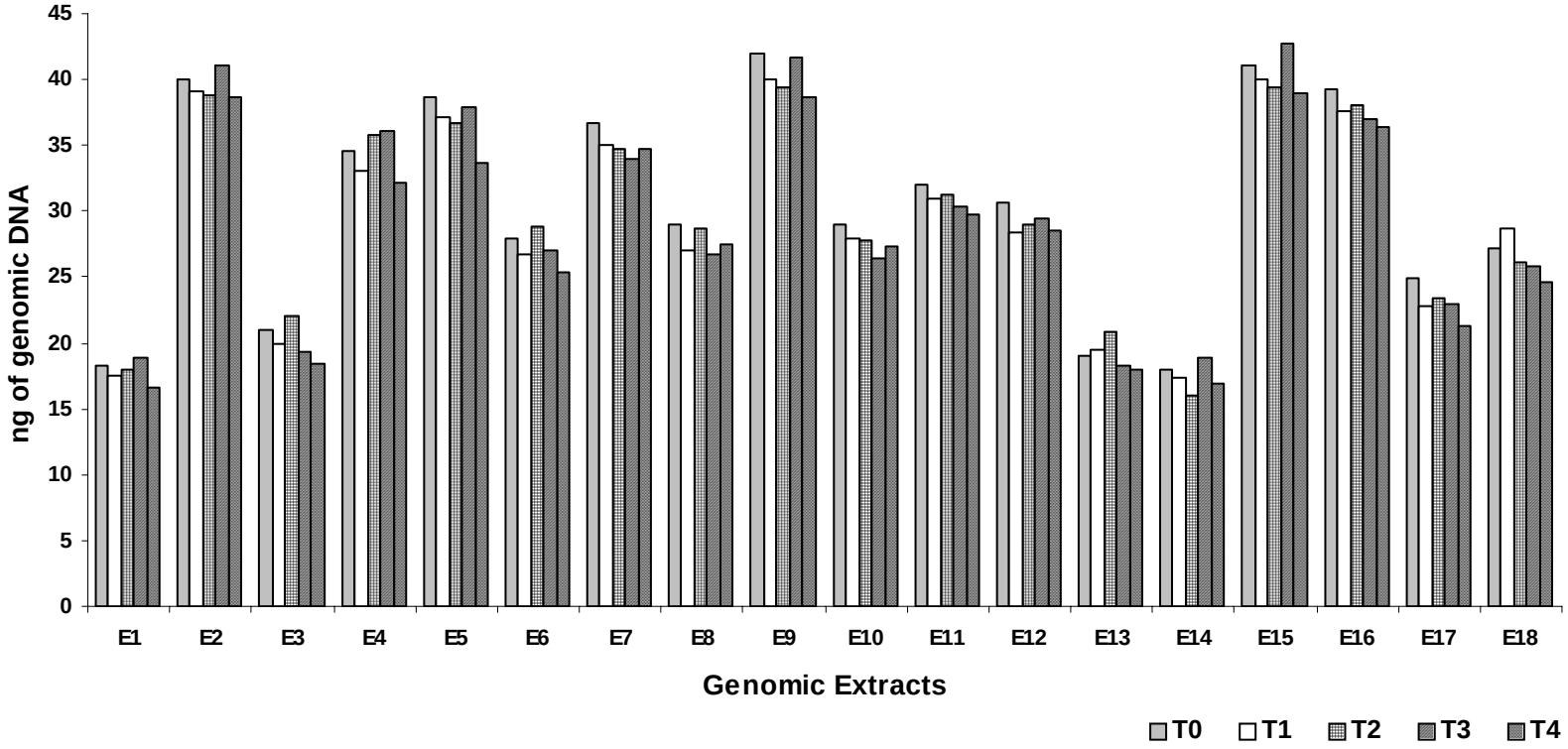
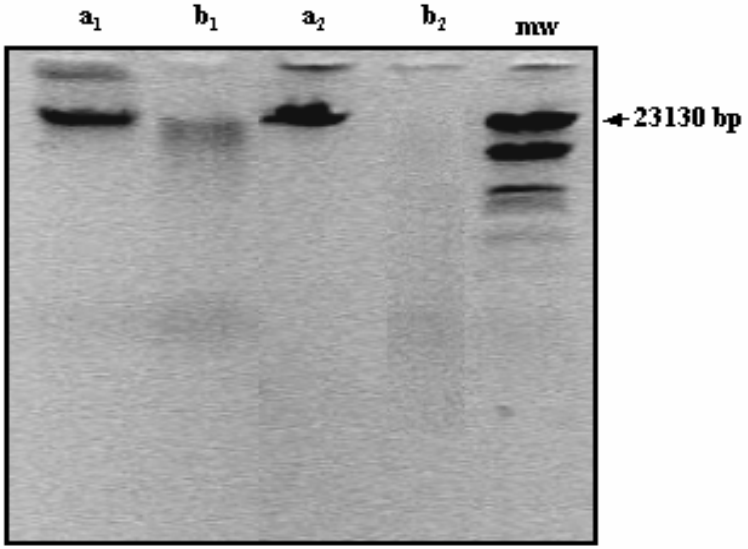


Figura 3. Variations in the DNA concentration in the extracts and times

Figure 4. Checking Gel of genomic DNA integrity.



ANEXO 3

PUBLICACIONES (en proceso de aceptación)

ANEXO 3.1

IN VITRO ASSESSMENT OF DNA DAMAGE AFTER CHRONIC EXPOSURE TO B(A)P USING RAPD AND THE RTG-2 FISH CELL LINE. (Enviado a la revista *ATLA*)

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IN VITRO ASSESSMENT OF DNA DAMAGE AFTER CHRONIC EXPOSURE TO B(A)P USING RAPD AND THE RTG-2 FISH CELL LINE.

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SUMMARY

Persistent and ubiquitous chemicals are nowadays sources of relevant environmental problems when considering the genotoxic effects associated with chronic exposure of natural populations. The high genetic similarity index between in vitro RTG-2 cells and in vivo rainbow trout individuals, showed by means of the RAPDs technique, allows to detect the genotoxic effects of chemicals by comparing DNA fingerprints from unexposed and exposed individuals. This study evaluates the capacity of RTG-2 cell lines together with the RAPD technique, in the detection of genotoxic effects on DNA after chronic exposure to B(a)P. RTG-2 cells were exposed for 3, 15 and 30 days to 0,05µg/ml B(a)P. After comparing control and exposed cells' DNA fingerprints both qualitatively and quantitatively, an increase in the instability of the exposed cells' DNA in a time dependent manner is shown. The sensitivity of the detection the sequence of primer used showing the existence of a B(a)P' hot spot in the DNA sequence. Two of the primers showed altered bands after only three days of exposure reaching 50% after the longest exposure period. Also the appearance of new bands was observed with a 100% level of reproducibility after 30 days of exposure. This work shows the usefulness of in vitro systems in population studies, considering cells as genetically homogenous populations, permitting us to undertake both acute and chronic studies, avoiding the inconveniences that studies in natural populations have.

Key words: Chronic effects, genotoxicity, fish cells, RAPD, DNA fingerprint, B(a)P.

INTRODUCTION.

Persistent and ubiquitous chemicals are nowadays sources of relevant environmental problems when considering natural populations. Environmental attacks diminish an ecosystem's ability to maintain productive and adaptable populations of organisms (1). Stochastic and deterministic demographic, environmental, and genetic factors contribute to a population's or a species' viability. Maintaining adequate levels of genetic diversity within and amongst populations is a critical aspect to consider (2).

Sustainable environmental development depends to a large extent on our capacity to monitor the effects that such chemicals may have upon ecosystems. Field studies have shown that continual exposure to certain contaminants produce a selective pressure on the affected populations, altering the alleles frequency of the most sensitive genotypes, and, a decrease in the reproduction and survival rate later on. (3). Moreover, laboratory toxicity studies show that chemically-induced mortality was dependent on genotype, suggesting that genotype frequencies are mediated by selection for certain alleles during toxicant exposure. (4, 5)

These types of effects have a great importance because they can manifest themselves long after the source of contamination has been eliminated (6).

A large number of biomarkers have been developed over the last couple of decades, in order to be employed in environmental monitoring studies. These biomarkers show the toxic effects caused by exposure to high concentrations of mutagenic and clastogenic chemicals to individuals, but uncertainty exists

concerning the potential genotoxic effects associated with chronic exposure to low levels of pollutants at a higher level of organization (population, community and ecosystem) (7)

Recent advances in molecular technology have opened a new chapter in the evaluation of a wide range of effects and in species conservation efforts.(2)

Allozyme variation (8, 9), nuclear or mitochondria restriction fragment length polymorphism (RFLP) (10, 11) DNA sequencing and Random Amplified Polymorphic DNA (RAPD) (12-14) are examples of these.

Nevertheless, in vivo chronic studies on populations are time consuming and expensive and involve such a high number and complexity of variables that the evaluation of results is very difficult. The studies carried out up till now have focused on almost confined populations, for which there were reliable controls available and a precise understanding of the studied contaminant. (1, 3, 15, 16).

Although the use of the in vitro tests are widely accepted in the testing sequence of genotoxicity of chemicals, this testing is used to assess effects at an individual level. However, these systems, formed by genetically homogeneous individuals in a controlled environment, can contribute to a better understanding of the genotoxic effects at a population level.

This population homogeneity linked to the choice of an adequate molecular technology have provided new tools for the detection of genetic alteration, by looking directly at the level of DNA sequence and structure of populations. RAPDs can be used for this purpose, because differences can be shown when comparing DNA fingerprints from unexposed and exposed individuals to genotoxic agents (7,17-24).

In previous works we have proved both the capability of rainbow trout derived cells, the RTG-2 cell line, and the RAPD technique to detect acute effects of known genotoxic chemicals (24). The high genetic similarity index between in vitro RTG-2 cells and in vivo rainbow trout individuals (25) allows this in vitro system, together with the RAPD technique, to show the genotoxic effects after chronic exposure periods of a known persistent water pollutant.

Polycyclic aromatic hydrocarbons (PAHs), chiefly produced by the use of fossil fuels, are ubiquitous at low concentrations and their presence in water during long periods of time, has been related with a high incidence of hepatic tumours in fish (26). Benzo(a)Pyrene is one such PAH and is classified by EPA as a priority pollutant due to its persistence, carcinogenicity, high bioaccumulation factor and biomagnification capability through the food chain. (27)

After being swallowed and metabolised by live organisms the B(a)P transforms itself into highly reactive chemicals one of which B(a)P-7,8-diol-9,10 epoxide metabolite (BPDE) is able to bind covalently to DNA and alkali-labile apurinic sites on DNA inducing mutations such as substitution of bases, frameshifts, insertions and deletions, some of which correlate with tumour initiation and progression. (28)

Some established cell lines derived from poikilothermic organisms have B(a)P metabolising ability (30). The RTG-2 cell line is one of them and can convert B(a)P to intermediate actives (31). For this reason this cell line has been used in cytotoxicity and genotoxicity studies after acute exposure to B(a)P. (32, 33)

In this work RTG-2 cells are exposed during 3, 15 and 30 days to low concentrations of B(a)P (0,05µg/ml) and its genotoxic action is measured using the RAPD technique.

MATERIALS AND METHODS

Cell Cultures

The RTG-2 fish cell line (34), an established fibroblastic cell line derived from rainbow trout (*Oncorhynchus mykiss*), was grown in Eagle Minimum Essential Medium with Earle's salts (EMEM), supplemented with 10% foetal serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 1.25 µg/ml fungizone, 2 mM L-glutamine and 1% non- essential aminoacids (complete medium) in PVC tissue culture flasks (Costar, Corning, NY) at 20 ± 1ºC in 5% CO2/air atmosphere. For cell subcultures, cells were detached from the substratum with trypsin-EDTA (Flow, Laboratories, Rickmansworth, UK), re-suspended in culture medium and split into three new flasks.

Toxic exposure

Cells, in their exponential growth phase, were exposed to 0.05 µg/ml, Benzo(a)Pyrene (B(a)P) (CAS nº 50-32-8, Sigma, USA) in complete medium for 3, 15 and 30 d. The selected concentration did not show genotoxic effects in acute assays with these same cells (35). Dimetil sulfoxide (DMSO) at 0.1% in complete medium was included as solvent control. A negative control (complete medium alone) was also included for each exposure period.

When cells reached confluence (after 15 and 30 days of exposure) flasks were subcultured at a 1:3 proportion. Exposure was contined in the same conditions in one of the subculture flasks and the other two are discarded.

On finishing each of the established exposure periods for the assay, the medium was removed and after being washed with phosphate-buffer saline (PBS, pH 7.3), the cells were detached from the substratum with trypsin-EDTA and collected in PBS in order for their subsequent analysis.

Two 75 cm2 flasks were used for the both control and exposed cells for each exposure period. The experiment was repeated on two different days.

DNA extraction and RAPD reaction

The DNA was extracted separately from each of the replicates by phenol-chloroform. Its integrity was checked by electrophoresis in 0.8% agarose gels using λ Phage DNA native as a molecular weight marker (Eurobio, Paris, France). The RAPD protocol was performed with 5 ng of DNA per reaction (36; 24) in a Perkin-Elmer model 2400 Thermalcycler, using four primers previously selected for their good *in vivo* / *in vitro* correlation (25). The primers were obtained from the Biopolymers Department of the CNBCR, (I.S. Carlos III, Majadahonda, Madrid). The selected primers were: 5´-GATCCATTGC-3´ (AA-82); 5´GGGCCTCTGAT-3´ (AA-89); 5´-AGCACTGTCA-3´ (C-96); 5´-CCAAGTCGACA-3´ (D-8)

Amplification products were resolved electrophoretically on 2.1% agarose gels and stained with ethidium bromide. The image was recorded by the Grab-it program (UVP, USA).

For greater accuracy in the comparison of the results both the control and the exposed DNA extract were run in the same gel. A negative control

reaction without DNA template, and a molecular weight marker (marker VI, Roche, Germany; or ϕX174-*Hae* III EUROBIO, France) were also included in the same gel. The bands were coded following the system described by Theodorakis and Shugart (1997) (3, 25)

Analysis of the results.

The image captured of each of the agarose gels was analysed by densitometry (Gelworks 1D, UVP, USA), and the fluorimetric profiles of each amplification reaction were studied both qualitatively and quantitatively.

Qualitative analysis was established by comparing the percentage appearance/absence of each of the bands present in the amplification profiles of the control and exposed DNA extracts.

Following the criteria established in a previous study, (24) the amplification bands for each of the primers were grouped together according to high, medium and low molecular weight in function of their amplification profile (Figure 1)

Quantitative analysis was performed taking as a comparative parameter the percentage of amplification of each of the bands, after the densitometric analysis of the control and exposed cells' profiles both in individual bands and when bands are grouped together.

The statistical tests used were the Student *t*-test and the Mann-Whinney *U*-test.

RESULTS

Both the quantitative analysis and the qualitative analysis show an increase in the instability of the exposed cells' DNA, depending on the toxic exposure period.

The sensitivity of the detection depended on the sequence of the primer used. The profiles that showed the greatest alterations were those that corresponded to the primers AA-89 and AA-82. Two of the bands (AA-89_{1225} and AA-82_{1075}) are significantly altered after only three days of exposure, with an increase in both primers the number of altered bands as the length of exposure increased, reaching close to 50 % after 30 days. (Table I)

The primer fingerprint C-96 is the most stable one, with one of the eight bands that is amplified (C-96_{1714}) showing significant statistical differences with respect to the controls at 15 and 30 days. The fluorimetric profile of the primer D-8, although remaining constant during the first two periods of exposure, alters drastically (up to the 40% of its bands) at 30 days of exposure. (Table I)

The time-dependent toxicant action of the B(a)P is clearly shown in the analysis of grouped bands with the primers AA-82 and AA-89. At 30 days of exposure both the high molecular weigh bands and those of low molecular weight are altered, presenting the former a decrease in their intensity of amplification, with those of medium and low molecular weight showing a proportional increase.(Figure 2)

In the qualitative study, the primer's fingerprints AA-82 and AA-89 were also the most affected by the toxicant, although terms, in general, the absence of bands of low fluorescence is observed also in a time-dependent manner (C-96_{1714}, AA-82_{700}). (Table II)

Perhaps the most outstanding result was the appearance, after 15 days of exposure, of new additional bands: D-8_{936}, AA-$82_{620,402}$ and AA-$89_{484.}$ Although the frequency of the appearance of these bands showed a very low reproducibility. (Figure 3)

In contrast to what was expected, when the exposure period was increased to 30 days the number of extra bands was reduced (AA-89_{484}, AA-82_{620}), but their reproducibility increased to 100% in all of the DNA extracts analysed. (Figure 4)

DISCUSSION

Environmental stress is just one of many potential causes for the reduction of a standing variation within a population. Altered genetic diversity was shown in fish populations living in sites affected by anthropogenic stressors, when compared with those living in unpolluted areas. The DNA-profile allows the detection of both chronic and acute exposure to stressors within populations with low migration rates, but the availability of reference populations is a prerequisite. (1, 3, 15, 16)

The need of control populations and the inherent difficulties associated with field studies (ranging from the climatic conditions of sampling, to the variety of size, age and sex of the individuals captured, etc.) limits the fulfillment of risk assessment studies in natural populations, particularly to evaluate chronic situations.

These problems can be minimized, in studies of the direct damage to DNA, using cell cultures from representative species of fish. Both due to the

strict control of the experimental conditions and the characteristics of a genetically homogenous population.

In vitro systems generate, by means of the RAPD technique, a specific and constant DNA fingerprint allowing for the detection of alterations in the DNA when comparing the fingerprints of control and exposed cells. The aforementioned alterations can be visualized in the DNA fingerprint as presence and/or absence of bands and/or alterations in their amplification intensity. (17-24).

This study evaluates the capacity of RTG-2 cell lines together with the RAPD technique, in the detection of chronic effects on DNA after exposure to B(a)P. The high level of similarity of this in vitro system with the species that it originates from, and the use of a set of primers previously selected for their good *in vivo-in vitro* correlation (25), allow to evaluate the before mentioned effects .

The sensitivity of he detection depends on the primer. Two of the four used (the AA-89 and AA-82) clearly show a greater alteration after the exposure to B(a)P, confirming the specificity of the mechanism of action this compound with a hot spot in the DNA sequence (28). Thus, after three days of exposure (1,5 cell cycles) and despite the RTG-2 cell line is able to convert 63% of the B(a)P to water-soluble metabolites (29-31) both qualitative and quantitative alterations are detected (Table I, II) The presence of bulky adducts that block the enzyme, and/or errors produced in the DNA replication are probably the cause of these early alterations. The mutation spectra resulting from DNA modifications by B(a)P are quite complex and depend on the sterochemistry of the adducts. These adducts have been shown to be capable of inducing base

substitution, frameshifts, insertions and deletions in specific sites of the DNA (28). These alterations can induce changes in the DNA sequence of a specific form generating new annealing primer-template sites.

Only when these alterations are present in sufficient numbers of cells, exceeding a mutation index of 10%, can they be seen in agarose gels (23). This would be in accordance with the presence of new bands in the amplification profiles originating from the cells exposed during 15 days. However, the nature of the RAPD's reaction, where the final products are the result of an exponential multiplication of the most abundant and stable fragments co-amplified in the first cycles, is the cause that these band ocasionaly are not reproductible in all cases. (37). (Figure 3)

Therefore, of the four amplified bands that appeared after 15 days of exposure two of them (AA-89_{484}, AA-82_{620}) not only persist after 30 days, but are present in 100% of the amplifications carried out. (Figure 4) This suggests as much the inheritability of aforementioned genetic alteration as an increase in the mutation index. These changes in the sequence, found in silent regions of the DNA, either transmit themselves to the daughter cells or are produced in sequences that codify vital proteins for the survival of the cells, in which case cytotoxic effects would be observed.

The genetic damage produced by the B(a)P increases in a time – dependent manner. Up to 50 % of the bands of the more sensitive primers are affected in the longest exposure period. In addition, a delay in the cell division (data not shown) has been observed. This has also been seen to in the same cells at a higher doses and with shorter exposure periods (35, 33)

Similar results were also observed using the same B(a)P concentrations (22) and other causative agents in different organisms, although both the protocol and the primer sequence were different (17-18, 20-23). In general, this loss of amplification is considered associated as a dose-dependent effect by the cited authors. Our results coincide in showing a time-dependent effect that is crucial for chronic assessment. Using the same chemical and the same cells we previously have demonstrated both a time-dependent and a dose-dependent effect of genotoxic action of B(a)P when measured by micronuclei induction (35)

In the primers where the sequences are more sensitive to the toxicant (AA-82 and AA-89) and when the damage to the DNA is great, the amplified fragments of high molecular weight show a reduction in their amplification intensity. (Figure 2) This could be a consequence of a greater possibility of DNA-toxicant interaction and of an additive effect of the damage caused (adducts, deletions etc). This same effect was already observed in previous work, using this same cell line, in acute exposures to mitomycin C. (24). These same primers are also those that in the studies of genetic variability carried out between the RTG-2 cell line and rainbow trout individuals showed the highest variability (>50%), suggesting the possibility that their sequences are more susceptible to alteration by external agents than other more conservative regions. (25).

CONCLUSIONS

It is clear that the combined use of in vitro systems and the RAPD technique permits us to detect alterations in the DNA originating in exposed organisms with a high degree of sensitivity. The alterations are detected in an

unspecific form by losses and / or gains of bands and variations in the amplification intensity.

When the objective is to establish if damage to DNA exists or not, i.e. for hazard identification in risk assessment studies, the presence in the fingerprint of any of these abnormalities would be enough show this effect.

This work shows the usefulness of in vitro systems for population studies, considering cells as genetically homogenous populations, permitting us to undertake both acute and chronic studies, avoiding the inconveniences that studies in natural populations have.

The high level of similarity of this in vitro study with the species from which the cells originate and the use of a set of primers previously selected for their good *in vivo-in vitro* correlation, permits the extrapolation of results and offers a cheap and versatile tool to evaluate the chronic effects of environmentally relevant compounds due to their persistence and ubiquity.

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Table I: Quantitative analysis of each of the bands obtained with the different primers, taking into account the variations in the percentages of the amplification, between control and exposed cells. The analysis has been undertaken considering the bands individually.

Bands	**3 d.** n = 12	**15 d.** n = 24	**30 d.** n = 24
AA-82$_{1075}$	**a**	**NS**	**a**
AA-82$_{1075}$	**NS**	**NS**	**NS**
AA-82$_{840}$	**NS**	**NS**	**NS**
AA-82$_{700}$	**NS**	**a**	**b**
AA-82$_{570}$	**NS**	**NS**	**NS**
AA-82$_{430}$	**NS**	**NS**	**a**
AA-89$_{1699}$	**NS**	**b**	**a**
AA-89$_{1566}$	**NS**	**NS**	**a**
AA-89$_{1418}$	**NS**	**NS**	**NS**
AA-89$_{1225}$	**a**	**a**	**a**
AA-89$_{1141}$	**NS**	**NS**	**NS**
AA-89$_{867}$	**NS**	**NS**	**NS**
AA-89$_{776}$	**NS**	**NS**	**a**
AA-89$_{463}$	**NS**	**NS**	**NS**
AA-89$_{450}$	**NS**	**NS**	**NS**
C-96$_{1714}$	**NS**	**b**	**c**
C-96$_{1321}$	**NS**	**NS**	**NS**
C-96$_{1099}$	**NS**	**NS**	**NS**
C-96$_{910}$	**NS**	**NS**	**NS**
C-96$_{856}$	**NS**	**NS**	**NS**
C-96$_{642}$	**NS**	**NS**	**NS**
C-96$_{487}$	**NS**	**NS**	**NS**
C-96$_{380}$	**NS**	**NS**	**NS**
D-8$_{1554}$	**NS**	**NS**	**c**
D-8$_{1172}$	**NS**	**NS**	**c**
D-8$_{1112}$	**NS**	**NS**	**NS**
D-8$_{825}$	**NS**	**NS**	**NS**
D-8$_{686}$	**NS**	**NS**	**NS**

NS = No significant differences versus control.

Significant differences versus control, a = P<0.05, b = P< 0.01, c = P< 0.001 by Mann-Whitney U-test. n number of replicate reactions.

Table II: Qualitative analysis: Bands presenting a difference between the control and exposed cells´ DNA fingerprint.

a)

Bands	Control	3 d.	Control	15 d	Control	30 d
	n =12		n =12		n =12	
AA-89$_{1225}$	**94**	**75**	**92**	**75**	**96**	**75**
C-96$_{1714}$	**100**	**100**	**100**	**72**	**100**	**65**
AA-82$_{700}$	**100**	**87**	**90**	**63**	**93**	**50**

b)

Bands	Control	3 d.	Control	15 d	Control	30 d
	n =12		n =12		n =12	
AA-89$_{484}$	**0**	**0**	**0**	**58**	**0**	**100**
D-8$_{936}$	**0**	**0**	**0**	**17**	**0**	**0**
AA-82$_{620}$	**0**	**0**	**0**	**42**	**0**	**100**
AA-82$_{402}$	**0**	**0**	**0**	**17**	**0**	**0**

a. *Bands that loose amplification in exposed cells at different periods of exposure.*

b. *New bands that appear in the exposed cells´DNA fingerprint.*

Data are expressed as percentage. n number of replicate reactions.

FIGURE LEGENDS

Figure 1.- Pattern for grouping of bands in function of the primer amplification profile.

HMW = high molecular weight, MMW = medium molecular weight, LMW = low molecular weight.

Figure 2.- Quantitative analysis for the bands obtained with the different primers, taking into account the variations in the percentages of the amplification, between control and exposed cells. The analysis has been undertaken considering the bands grouped together in high, medium and low molecular weight bands, for each of the primers.

Figure 3.- Agarose gels showing the DNA band patterns obtained with the different primers (AA-82, D-8, AA-89 and C-96). DNA extract from control (c) and from treated cells (3, 15 and 30) after different exposure periods (days). Arrows show the appearance /disappearance of bands. (mw) molecular weight marker. (w) negative control reaction without DNA template.

Figure 4.- Agarose gels showing the reproducibility of the appearance of the extra bands on the DNA fingerprint cells after been treated for 30 days obtained with the AA-82 and AA-89 primers. **(C)** DNA extract from control cells: (nc) negative control – complete medium- and (sc) solvent control – complete medium with solvent. **(30 d)** DNA extracts from exposed cells at 30 days (E1 and E2). Arrows show the appearance of the new bands: AA-89_{484} y AA-82_{620}. (mw) molecular weigh marker. (w) negative control reaction without DNA template.

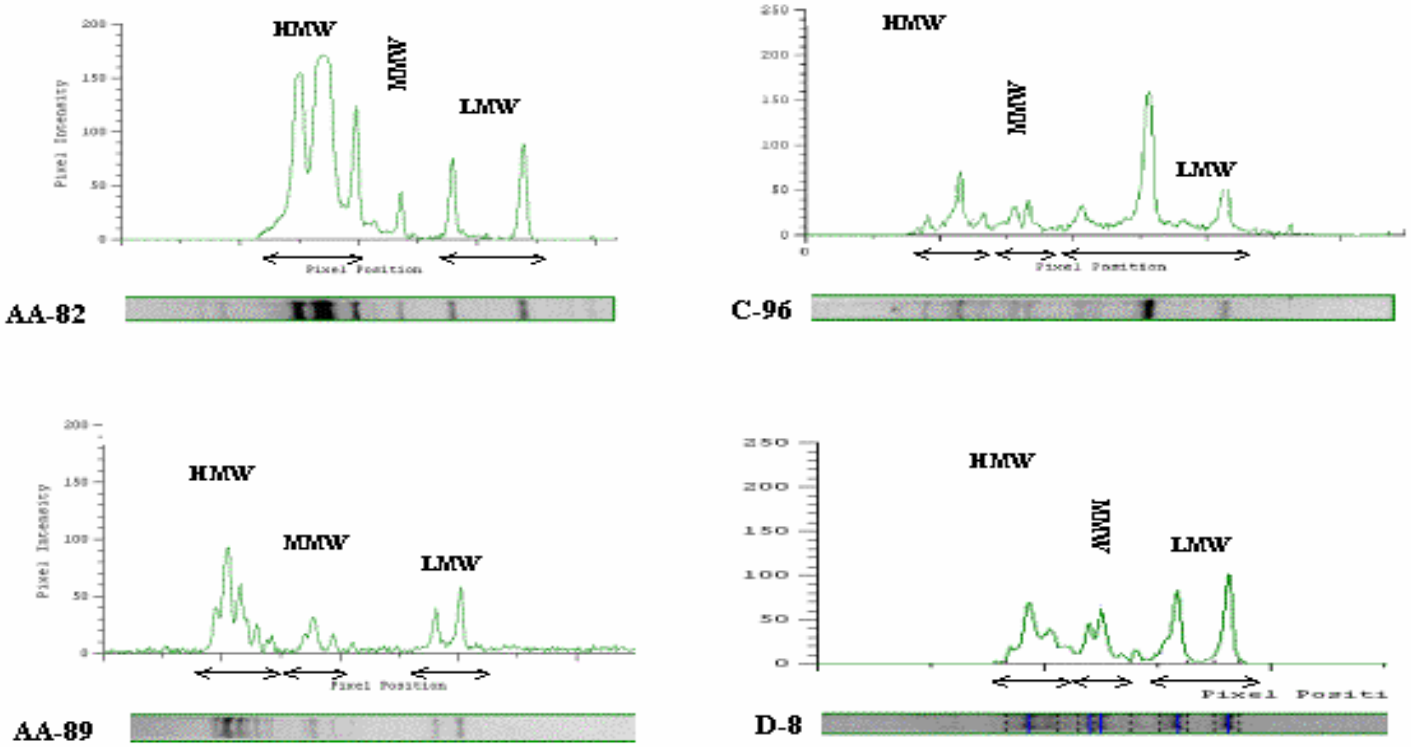

Figure 1

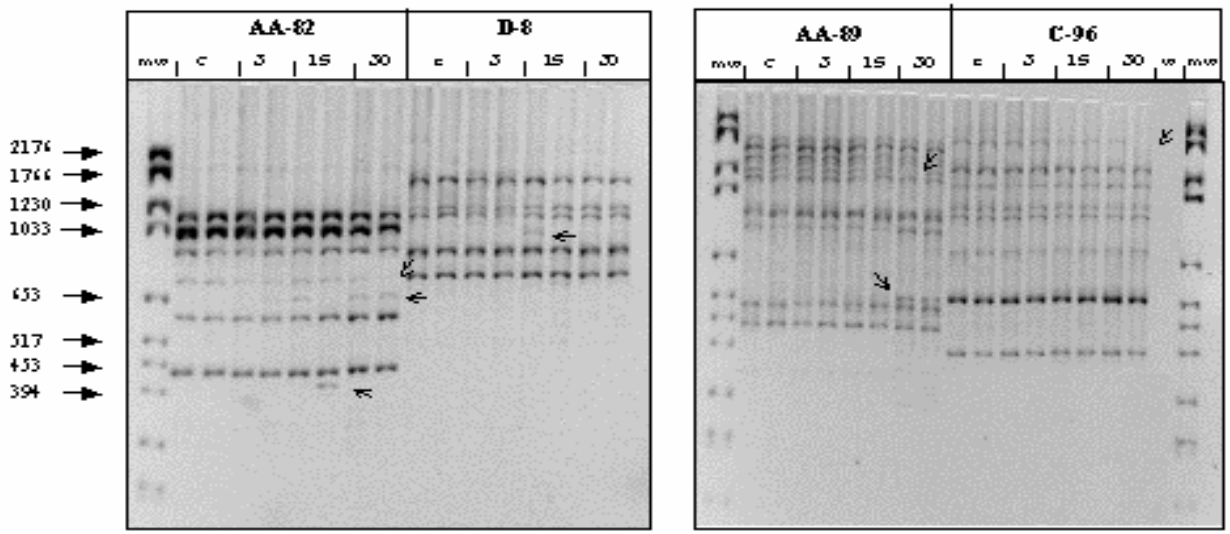
AA-82
D-8
AA-89
C-96
mw
c
3
15
30
e
w
2176
1766
1230
1033
653
517
453
394

Figure 2

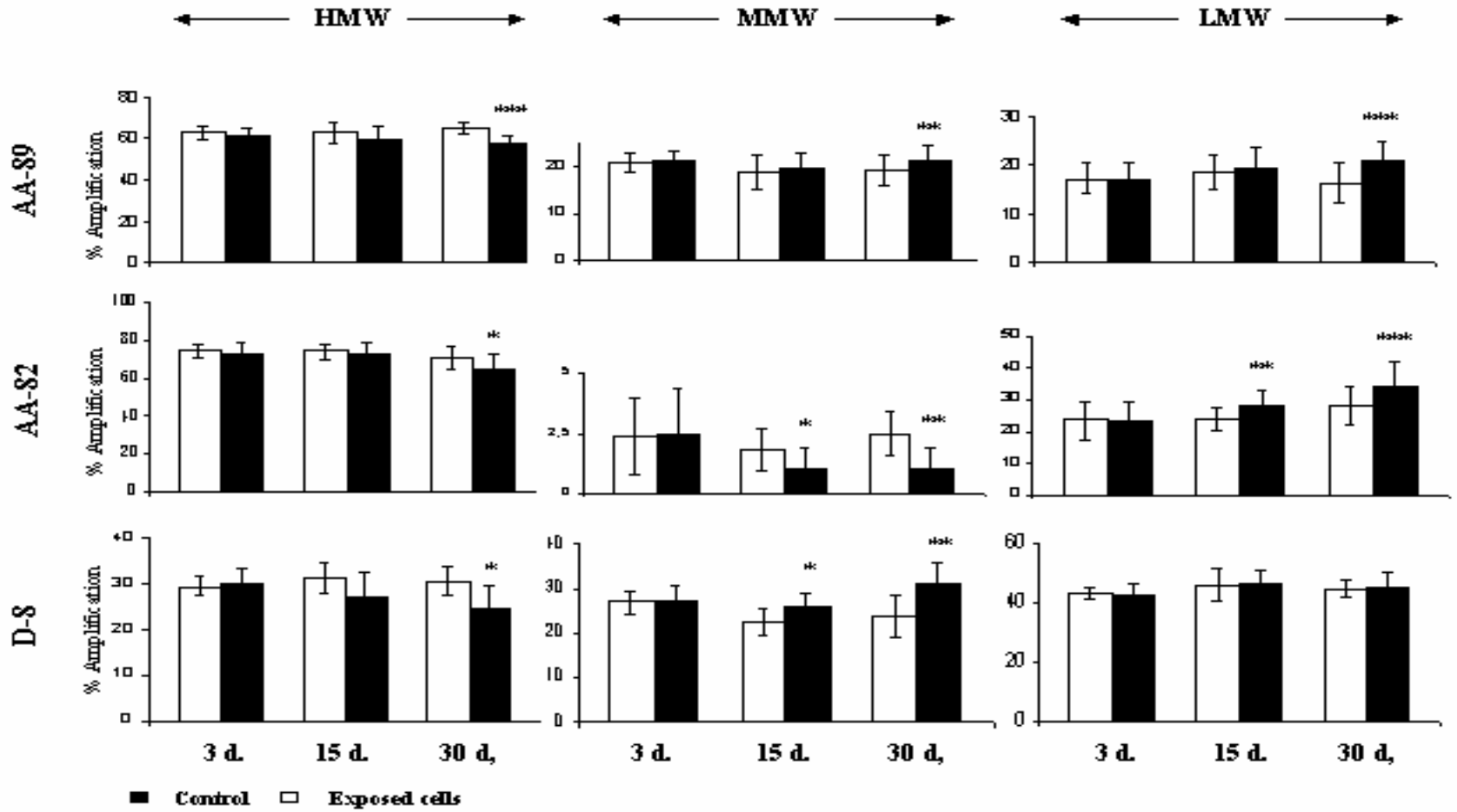
HMW
MMW
LMW
AA-89
AA-82
D-8
% Amplification
3 d.
15 d.
30 d,
Control
Exposed cells

Figure 3

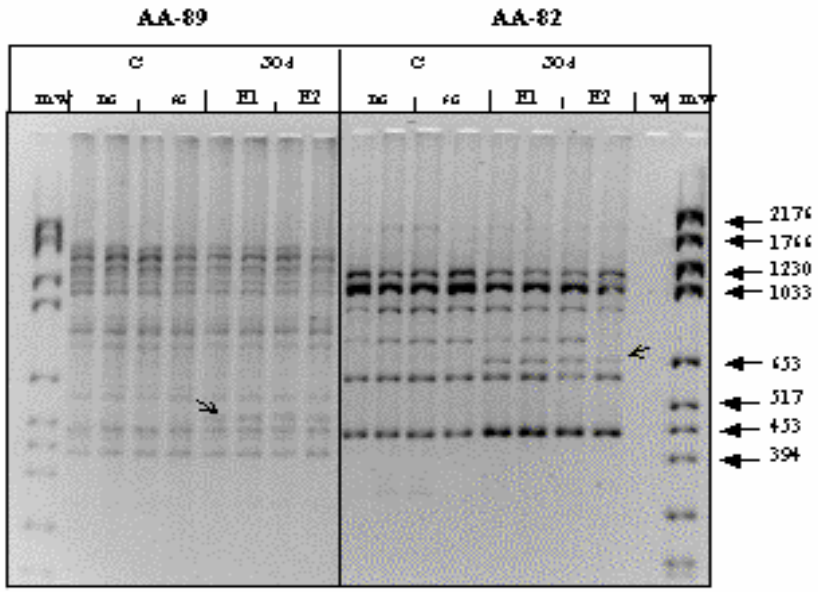
AA-89
AA-82
C
304
C
304
mw
ng
sg
E1
E2
ng
sg
E1
E2
w
mw
2176
1766
1230
1033
653
517
453
394

Figure 4

ANEXO 3.2

IN VITRO DETECTION OF ALTERATIONS IN THE DNA AFTER ACUTE EXPOSURE TO B(a)P USING THE RAPD TECHNIQUE. (Enviado a la revista CHEMOSFERE)

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IN VITRO DETECTION OF ALTERATIONS IN THE DNA AFTER ACUTE EXPOSURE TO B(a)P USING THE RAPD TECHNIQUE.

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SUMMARY

Several factors are involved in the loss of the world's biodiversity. Environmental genotoxins are often associated with the decline or disappearance of many populations. Sensitive and specific tests are required to safely establish the security levels of chemicals prior to their use, or for hazard assessment. The use of *in vitro* systems, i.e. cells in culture, together with the recently developed molecular techniques, are promising tools for this objective. In this work the sensitivity of the Random Amplified Polymorphic DNA technique is studied, to detect the deleterious effect of a known pro-mutagen, B(a)P, after the exposure of an established fish cell line, RTG-2, to 0.05, 0.1, 0.5 µg/ml for 24, 48 and 72 h. The concentration and length of exposure are inversely related, confirming the results previously observed using the same cell line, chemical and exposure periods, but measuring the increase of micronuclei frequency. In identical conditions, double of the concentration of chemicals is necessary (0.1 µg/ml) in order to detect the presence of micronuclei and lower concentrations of B(a)P (0.05 µg/ml) alter the cells' DNA-fingerprint after 72 hours of exposure. This study shows that the RAPD technique presents a high level of sensibility in the early detection of DNA damage produced by this relevant environmental chemical. Although these results have to be confirmed with a higher number of chemicals, its application can be of great usefulness in the detection of the genotoxicity of potentially dangerous substances for aquatic biota.

Key words: Fish cell line, Acute effects, Genotoxicity, RAPD, DNA fingerprint, B(a)P.

1. INTRODUCTION.

Ecosystems result from the dynamic interactions of living and inert matter where the living material acclimatizes and adapts to environmental change (Shugart and Theodorakis, 1996).

Pollution, in general, can represent a stress factor selectively leading to a change in the genetic make-up. In addition, environmental genotoxins can directly alter gene pools. A change in the genetic constitution may be advantageous for certain populations living in stressful conditions, but may present a disadvantage for others, including man. (Wurgler and Kramers, 1992)

Several factors are involved in the loss of the world's biodiversity. Chemical contamination of the environment, including the effect of long-term, low level chronic exposure of populations, as well as acute exposures have been implicated in the decline or disappearance of many populations. (Bickham et al., 2000). The genetic apparatus of an organism can interact with genotoxicants in a variety of ways and an understanding of the cellular mechanisms involved in these interactions provide the researcher with the opportunity to predict, and possibly prevent, contaminant-induced genetic damage in exposed populations. (Shugart and Theodorakis, 1996).

Obviously both the exposure and the nature of the chemical are key factors in the observed cellular effects, therefore precise information (dose-response data) is required in hazard identification as a requisite for a correct risk assessment and to establish the adequate security factors.

Genotoxicity detection techniques both in field and laboratory studies were based until a few years ago on the application of the classic assays of genotoxicity used in human risk assessment. (Wurgler and Kramers, 1992).

The most commonly used assays: exchange of sister chromosomes, detection of chromosomic aberrations etc. involve the observation of the chromosomes through an optic microscope. However, when fish species are used problems are presented that are associated with the small size of the chromosomes and the low metabolic rate of the fish. (Sugg, et al., 1996).

However, advances in molecular biology have led to the development of a number of selective and sensitive assays for DNA analysis: oncogene activation (Abshire et al., 1996), the use of transgenic animals (Murti et al., 1994), detection of micronuclei by flow cytometry (Sanchez et al., 2000; Grawe et al., 1993), a molecular marker for the detection of genetic alteration by looking directly at the level of the DNA sequence and structure (Jeffreys, 1987). These new techniques have considerable promise for increasing our understanding of both mechanisms of toxicity on genomes or genome products and the relevance of detrimental effects to individual fitness. (Anderson et al., 1994.)

Random Amplified Polymorphic DNA (RAPD) developed by Williams et al. (1990) is one of those. Based on the classic PCR, it uses low temperatures of annealing and short primers of arbitrary sequence. This allows to scan the DNA molecule. The final result is the amplification of a determined number of anonymous segments that, after being revealed in agarose gels, generate a constant band fingerprint specific to the genome used.

This technique doesn't require an exact understanding of the amplification sequence, and the same primer can be used in different species for the detection of polymorphisms. These characteristics have made it very useful in studies directed at genetic populations (Welsh et al.,1991), identification of species (Koh et al., 1998; Szalai et al., 1997; Henry et al., 1997), construction of genetic maps (Al-Zahim et al., 1997), and for the detection of the genotoxic effects on an individual and / or population level (Kubota et al., 1995; Conte et al.,1998; Atienzar et al., 1998; 1999; 2000; Becerril et al., 1999; Theodorakis et al.,1997; Krane et al.,1998).

Obtaining a specific DNA-fingerprint permits a comparison between the control and exposed individuals to detect changes in the DNA. The use of a high number of different primers increases the sensitivity in the detection of alterations in the genomic DNA.

This technique had previously been put in doubt due to the lack of reproducibility, and the appearance of erratic bands that complicate the analysis of the results (Penner et al.,1993; Meunier and Grimont,1993; Ellsmorth et al., 1993; Davin-Regli, 1995). Although it is worth noting that this technique is very sensitive to protocol changes, when the protocol has been meticulously established the results are shown to be highly repetitive (Atienzar et al., 2000b).

The application of this technique in biological genetically homogenous systems , such as cell cultures, also presents great advantages: testing a large number of samples, the use of a low chemical volume, reduced wastes and the possibility to use a high number of cells simulating the effects on an exposed population. Finally, after comparing the DNA fingerprint of the control and

exposed cells alterations can be detected in the genomic material produced by the toxin.

The RTG-2 cell line proceeding from the rainbow trout has been used extensively in *in vitro* studies in order to evaluate the cytotoxicity and genotoxicity of environmental samples. (Babich et al., 1995) This cell line also presents a good *in vivo* / *in vitro* correlation, both in its capability to predict acute toxicity (Castaño et al., 1994; 1996) as in its genetic fingerprint. It is also particularly interesting in genotoxicity studies in that it is capable of metabolising compounds without adding to the average systems of metabolic activation. (Thornton et al., 1982; Clark and Diamond, 1970; Ajaujo et al., 2000)

In previous work we have established an optimum protocol for the detection of alterations in the DNA fingerprint of this cell line after exposure to the reference genotoxic. Also in *in vivo* / *in vitro* studies, we established the intimate genetic inter-relation that exists between both systems, permitting the extrapolation of the results of genotoxicity in a theoretic form. (Becerril et al., 2001)

The object of this work is to evaluate the sensitivity offered by RAPD technique in comparison with another technique whose endpoint is widely accepted, the increase of the frequency of the micronuclei.

In the current work the genotoxic effects of acute exposure to B(a)P are studied, on RTG-2 cells evaluated using the RAPD technique and using primers previously selected for their good *in vivo* / *in vitro* correlation, and these results will be compared with those obtained by using the detection of micronuclei by flow cytometry after exposing this same cell line in the same conditions (Sánchez et al., 2000).

2. MATERIAL AND METHODS

The RTG-2 cell line, an established fibroblastic-like cell line (ATCC CCL 55) derived from rainbow trout gonadal tissue (*Oncorhynchus mykiss*) (2n= 60) was used in this study.

RTG-2 cells were grown on Minimal Essential Medium (MEM-Earle) supplemented with 10% of foetal bovine serum (FBS), penicillin (0.05 IU/ml), streptomycin (0.05 μg/ml), amphotericin B (1.25 μg/ml), glutamine (2mM) and 1% non-essential monoacids, at its optimum grow temperature 20º± 1º C, pH 7.3 and 5% CO_2 in air.

2.1. Chemical Exposure

Benzo(a)pyrene (B(a)P) (CAS nº 50-32-8, Sigma, USA) was first dissolved in dimethyl sulfoxide (DMSO) at a concentration of 1 mg/ml. B(a)P was assayed at concentrations of 0.05, 0.1 and 0.5 μg/ml for 24, 48 and 72 h . The solvent (DMSO), never exceeded 0.1% v/v.

Each concentration and exposure period was replicated once. Experiments were repeated in different days (4 replicates). Two negative controls, a MEM control and a solvent (DMSO) control were included in each exposure period. After treatment, medium with toxic was removed, cultures were washed with Phosphate Buffered Saline (PBS), and fresh medium was added. Cultures with fresh medium were left to grow for 72 h (including the exposure period).

2.2. DNA extraction and RAPD reaction

Cells were dissociated with tripsin-EDTA (Flow, Laboratories, Rickmansworth, UK), collected in PBS, pH 7.3 and their DNA isolated by phenol-chloroform extraction. The integrity of extracted genomic DNA was checked by electrophoresis in 0.8% agarose gels using λ Phage native DNA as molecular weight marker (Eurobio, France).

The RAPD reaction previously described (Ferrero et al., 1998; Becerril et al., 1999) was performed in a total volume of 25 µl volume containing 4 mM $MgCl_2$, 2 U Stoffel Fragment (Perkin-Elmer, Branchburg,NY), 0,2 mM each dNTP (Pharmacia, Barcelona, Spain) and 5 ng DNA template. Temperature cycle was: an initial denaturing step at 92ºC followed by 45 cycles of annealing at 36ºC for 75 s and extension at 72ºC for 6 min. The thermalcycler used was a Perkin-Elmer model 2400.

Four primers previously selected for their good *in vivo* / *in vitro* correlation were used in this study (Becerril et al., 2001). Their sequences are, AA-82: 5´-GATCCATTGC-3´; AA-89: 5´GGGCCTCTGAT-3´; C-96: 5´-AGCACTGTCA-3´; D-8: 5´-CCAAGTCGACA-3´. The primers were obtained from the Biopolymers Department of the CNBCR, (I.S. Carlos III, Majadahonda, Madrid).

Amplification products were resolved electrophoretically on 2.1% agarose gels and stained with ethidium bromide. The image was recorded using the Grab-it program (UVP, USA).

The amplifications were carried out in duplicate and on different days for each of the primers used.

In each gel and for a better comparison of the results, control and exposed cells were run at the same time. A negative control reaction without

template DNA, and a molecular weight marker (marker VI, Roche, Germany) were also included in the same gel. The bands were coded following the system described by Theodorakis and Shugart (1997).

2.3. Analysis of the results.

The image captured of each of the agarose gels was analysed by densitometry (Gelworks 1D, UVP, USA), and the fluorimetric profiles of each amplification reaction were studied both qualitatively and quantitatively.

Qualitative analysis was established by comparing the percentage appearance / absence of each of the bands present in the amplification profiles of both the control and exposed DNA extracts.

Qualitative analysis was established by comparing the percentage appearance/absence of each of the bands present in the amplification profiles of the control and exposed DNA extracts.

Following the criteria established in previous studies, (Becerril et al., 1999) the amplification bands for each of the primers were grouped together according to high, medium and low molecular weight according to their amplification profile (Fig. 1)

Quantitative analysis was performed taking as a comparative parameter the percentage of amplification of each of the bands, after the densitometric analysis of the control and exposed cells' profiles both in individual bands and when bands are grouped together.

The statistical differences were established using the Student *t*-test and the Mann-Whinney *U*-test.

3. RESULTS.

After comparing the amplification profiles from the control and exposed cells, we can observe both quantitative differences (Table I), evaluated using the amplification percentage, and qualitative differences, expressed as absence or presence of new bands (Table II).

Figure 2 shows graphically only those bands which present significant statistical differences with respect to the control, for each of the concentrations and exposure periods used in the assay.

The lowest concentration tested (0.05 µg/ml) produced significant alterations after the longest exposure period (72 hours). In these conditions 7.1% of the bands are shown to be altered. However, when the concentration is increased to 0.1 and 0.5 µg/ml these effects show themselves in shorter periods, 7.1% and 28.6% respectively of the total of the bands after 24 hours of exposure. These percentages increase to 42.8% and 53.6% of the bands when either the concentration and/or the period of exposure are at their maximum.

Analysing the results of each of the primers individually one can observe differences in their sensitivity against the action of the toxin. The primers AA-89 and AA-82, in the lowest concentration used in the assay, show altered profiles (bands AA-82_{1075} and AA-89_{1225}), while the amplified fragments with the primer C-96 remain stable, only showing alteration at higher concentrations and the longest exposure time (Fig. 3).

When the high, middle and low molecular weight bands are grouped together, their amplification percentages show values that are statistically significant after periods of exposure of 48 and 72 hours, but chemical

concentrations greater than 0.05 µg/ml are necessary. In general an inverse relation is observed between the amplification intensity of the different fragments and their molecular weights (Fig. 4).

The qualitative study shows that the exposed cells' DNA-fingerprint with respect to the control cells, presents an absence of bands in some of the amplifications. The percentage of disappearance of the aforementioned bands is directly proportional to the concentration and time of exposure to the toxin. So, when both parameters are at their maximum, all the primers present anomalous amplifications with absences in some of their bands (Table II). The earliest effects are observed in the bands AA-89_{1225} and AA-82_{700} only present in 76% and 62% of the amplifications after a 24 h period of exposure to 0.5 µg/ml of toxin.

Figure 5 shows the presence of three new bands in the fluorimetric profiles of the AA-82 and D-8 primers (AA-82_{620}, D-8_{936}, D-8_{798}). These new bands are only visible after long periods of exposure and when the toxin concentration is 0.05 and 0.1 µg/ml of B(a)P.

4. DISCUSSION.

Cell cultures offer great advantages against *in vivo* studies in the detection of damage to the DNA. Apart from the inherent advantages of easy handling, low cost and a precise control of the environmental conditions, for genotoxicity studies they present a constant and specific DNA-fingerprint, avoiding the presence of polymorphisms common to *in vivo* systems.

The reproducibility of the DNA-fingerprint, generated by RAPD was initially put in doubt by the sporadic appearance of erratic bands, in some cases

difficult to interpret (Davin-Regli, 1995; Penner et al., 1993). However when the reaction is meticulously standardized the results are reliable and repetitive. This way the changes produced in the amplification profile are attributed to modifications in the DNA template (Kubota et al., 1995; Atienzar et al., 2000; Becerril et al, 1999). Moreover, when the extraction technique is adequate, the extracts can be stored until their use, during relatively long periods of time. (Becerril et al., in press).

The purpose of this work is to compare the sensibility the detection of DNA damage offers, with a more classic and widely accepted methodology such as the detection of micronuclei (Nüsse et al., 1994).

The observed effects are time and dose dependent, confirming the results obtained in a previous study with the same cell line, the same chemical concentrations and exposure periods used in this work (Sánchez et al., 2000). However, the RAPD technique shows greater sensitivity. The results obtained show that low concentrations of B(a)P (0.05 µg/ml) alter the cells' DNA-fingerprint after 72 hours of contact with the toxin (1.5 cycles). In identical conditions, double the concentration of toxin is necessary (0.1 µg/ml) in order to detect the presence of micronuclei. (Sánchez et al., 2000).

Results observed by other authors through the assessment of anaphase chromosome aberrations (Kocan et al., 1982) or alterations in the variation coefficient DNA content in the G1 phase using the same cell line also show lower sensitivity (Kocan et al., 1985).

Although all these techniques detected DNA damage, the presence of micronuclei, and / or chromosome aberration reveal breaks in the DNA, whilst the RAPD technique detects an extensive and varied spectre of alterations in

the genetic material (Kubota et al., 1995; Atienzar et al., 1999). This would justify its greater capacity in the detection of DNA damage at lower concentrations of the toxin.

The most frequently detected changes in the DNA fingerprint are shown as percentage differences of amplification and loss of amplification of some bands, generally of low intensity. Both can be the result of a combination of events, one of the earlier effects being the formation of adducts. These can block the enzyme action and alter the amplification process in function of the toxin's concentration. It's probable that this is the cause of the alterations detected after 24 h of exposure.

The bands of greater molecular weight are those that are most easily affected, which to be expected since a greater length increases the probability of interaction between the DNA and the toxin (Becerril et al.,1999).

Other events, such as small deletions or insertions or changes in the structure of the chromosomes due to the formation of breaks and / or reorganizations, can be the cause of the disappearance and/or appearance of new bands and affect the amplification intensity. Some places of primer-template union can disappear in them or even other new can be created. This could be the cause of the appearance of the new bands AA-82_{620} and D-$8_{936,798}$ or of the disappearance of the bands D-8_{1554} and AA-82_{700} in more than 50% of the amplifications after 72 hours of exposure. The absence or presence of bands is a less frequent phenomenon than the variations in fluorimetric intensity since it requires that a same event affects a relatively high number of cells in order that it becomes visible in agarose gels (not less than 10%) (Atienzar et al., 2000).

Other alterations such as mutations due to changes of bases or faults in the replication of the DNA could be detected using this technique.

Not all the primers present amplification profiles sensitive to the action of the toxin. Whilst the primer C-96 remains unalterable except at the greatest concentration and time of exposure, others (AA-82 and AA-89) present early alterations in some of their bands confirming the great affinity of the toxin towards certain DNA sequences. The specific action of the B(a)P on certain DNA sequences could be the cause of these results. If this were the case the sequences presented by the bands affected earliest (AA-82 and AA-89) would contain a hot spot to the toxin's action, (Bigger et al., 2000) and their sequencing would help to increase our comprehension of the mechanisms of the B(a)P action.

The length of exposure period and the concentration of B(a)P affect metabolic efficiency, or cell growth (Kocan et al., 1982). Using flow cytometry, cell cycle alterations are shown in RTG-2 cells after 72 h of exposure to 0.5 µg/ml of the chemical (Sánchez et al., 2000). In these conditions the RAPD technique shows high levels of DNA damage that could possibly correspond to the presence of a cytotoxic process: alterations in the amplification intensity (56.6% of the total) and, in general, an increase in the disappearance of bands. The presence of new bands only occurs at this stage of exposure (72 h), but at lower toxin concentrations (0.05 and 0.1 µg/ml). The alterations in the cell cycle delaying cell division (Araujo et al., 2000) and the appearance of cytotoxicity at high toxin concentrations can be the cause of new bands not appearing at this concentration.

This study shows that the RAPD technique presents a higher level of sensitivity in the early detection of DNA damage when compared to other well established genotoxicity assays. Its use together with cell lines, capable to metabolise relevant environmental chemicals, can be of great usefulness in the detection of the genotoxicity for aquatic biota.

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Table I. Quantitative analysis of each of the bands obtained with the different primers, taking into account the variations in the percentages of the amplification, between control and exposed cells to different exposure periods and B(a)P concentrations.

	24 h			48 h			72 h		
	0,05	0,10	0,50	0,05	0,10	0,50	0,05	0,10	0,50
		n=16			n=16			n=16	
AA-82$_{1075}$	ns	ns	a	ns	ns	c	a	ns	ns
AA-82$_{965}$	ns	ns	ns	ns	ns	c	ns	c	c
AA-82$_{840}$	ns	ns	ns	ns	ns	ns	ns	ns	ns
AA-82$_{700}$	ns	ns	c	ns	c	c	ns	c	c
AA-82$_{570}$	ns	ns	ns	ns	a	c	ns	c	c
AA-82$_{430}$	ns	ns	c	ns	ns	c	ns	ns	c
D-8$_{1554}$	ns	a	c	ns	ns	c	ns	c	c
D-8$_{1172}$	ns	ns	a	ns	a	c	ns	b	b
D-8$_{1112}$	ns	ns	ns	ns	ns	ns	ns	ns	ns
D-8$_{825}$	ns	ns	ns	ns	ns	ns	ns	b	b
D-8$_{686}$	ns	ns	a	ns	ns	c	ns	ns	c
C-96$_{1714}$	ns	ns	ns	ns	ns	ns	ns	ns	a
C-96$_{1321}$	ns	ns	ns	ns	ns	ns	ns	ns	ns
C-96$_{1099}$	ns	ns	ns	ns	ns	ns	ns	ns	a
C-96$_{910}$	ns	ns	ns	ns	ns	ns	ns	a	a
C-96$_{856}$	ns	ns	ns	ns	ns	ns	ns	ns	ns
C-96$_{642}$	ns	ns	ns	ns	ns	ns	ns	ns	ns
C-96$_{487}$	ns	ns	ns	ns	ns	ns	ns	ns	ns
C-96$_{380}$	ns	ns	ns	ns	ns	ns	ns	ns	ns
AA-89$_{1699}$	ns	ns	b	ns	b	c	ns	c	b
AA-89$_{1566}$	ns	ns	ns	ns	ns	c	ns	c	b
AA-89$_{1418}$	ns	ns	ns	ns	ns	ns	ns	ns	ns
AA-89$_{1225}$	ns	a	ns	ns	a	b	a	b	a
AA-89$_{1141}$	ns	ns	b	ns	a	b	ns	b	c
AA-89$_{867}$	ns	ns	ns	ns	ns	ns	ns	ns	ns
AA-89$_{776}$	ns	ns	ns	ns	ns	ns	ns	ns	ns
AA-89$_{463}$	ns	ns	ns	ns	ns	c	ns	c	c
AA-89$_{450}$	ns	ns	ns	ns	a	c	ns	c	c

ns = No significant differences versus control. Significant differences versus control, a = P<0.05, b = P< 0.01, c = P< 0.001 by Mann-Whitney U-test
n number of replicate reactions.

Table II: Qualitative analysis. Bands presenting a difference between the control and exposed cells´ DNA fingerprint.

a.

	24 h				48 h				72 h			
Bands	**C**	**0.05**	**0.1**	**0.5**	**C**	**0.05**	**0.1**	**0.5**	**C**	**0.05**	**0.1**	**0.5**
AA-89$_{1225}$	**100**	**100**	**100**	**76**	**100**	**100**	**83**	**67**	**100**	**100**	**50**	**58**
AA-89$_{1141}$	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**81**	**100**	**100**	**67**	**50**
AA-82$_{700}$	**100**	**100**	**100**	**62**	**100**	**100**	**67**	**56**	**100**	**100**	**67**	**42**
D-8$_{1554}$	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**92**	**100**	**100**	**100**	**48**
C-96$_{1714}$	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**71**	**75**

b.

	24 h				48 h				72 h			
Bands	**C**	**0.05**	**0.1**	**0.5**	**C**	**0.05**	**0.1**	**0.5**	**C**	**0.05**	**0.1**	**0.5**
D-8$_{936}$	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**6.5**	**25**	**8.3**
D-8$_{798}$	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**6,5**	**12.5**	**0**
AA-82$_{620}$	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**25**	**0**

a. *Bands that lose amplification in exposed cells at different periods of exposure.*

b. *New bands that appear in the exposed cells´DNA fingerprint.*

Data are expressed as percentage. 16 replicate per reaction. C control

FIGURE LEGENDS

Figure 1.- Pattern for grouping of bands in function of the primer amplification profile.

HMW = high molecular weight, MMW = medium molecular weight, LMW = low molecular weight.

Figure 2.- The total bands with significant statistical differences respect to the control for each of the concentrations (0.05, 0.10, 0.50 µg/ml) and exposure periods used in the assay (24, 48, 72 h).

Figure 3.- Percentage of altered bands generated for each primers respect to the control to different concentrations (0.05, 0.10, 0.50 µg/ml) and exposure periods used (24, 48, 72 h).

Figure 4.- Quantitative analysis for the bands obtained with the different primers, taking into account the variations in the percentages of the amplification, between control and exposed cells. The analysis has been undertaken considering the bands grouped together in high, medium and low molecular weight bands, for each of the primers to different exposure periods and B(a)P concentrations.

Figure 5.- Agarose gels showing the DNA band patterns obtained with the AA-82, D-8 primers. DNA extract from control (C) and from treated cells (0.05, 0.1 and 0.5) after different exposure periods (24, 48 and 72 h). Arrows show the appearance /disappearance of bands. (mw) molecular weight marker. (w) negative control reaction without DNA template.

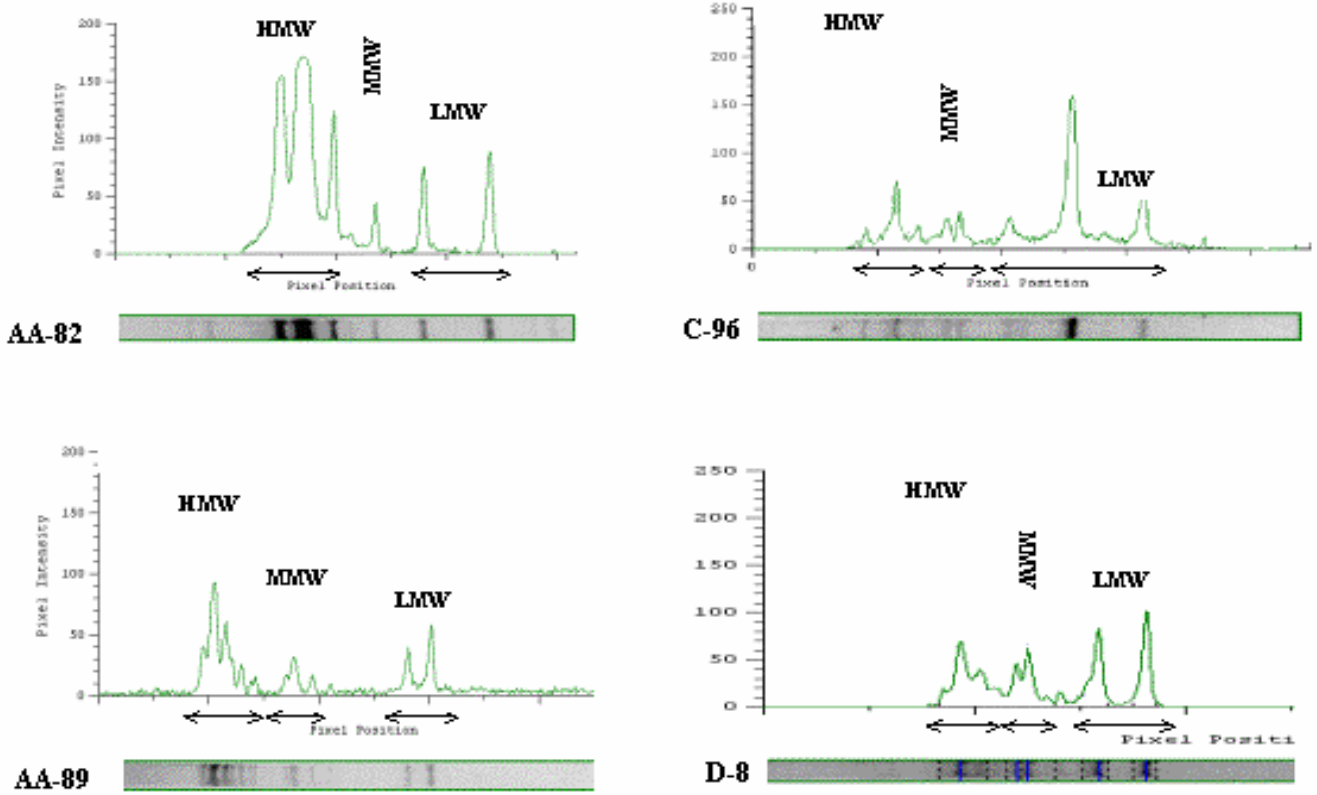

Figure 1

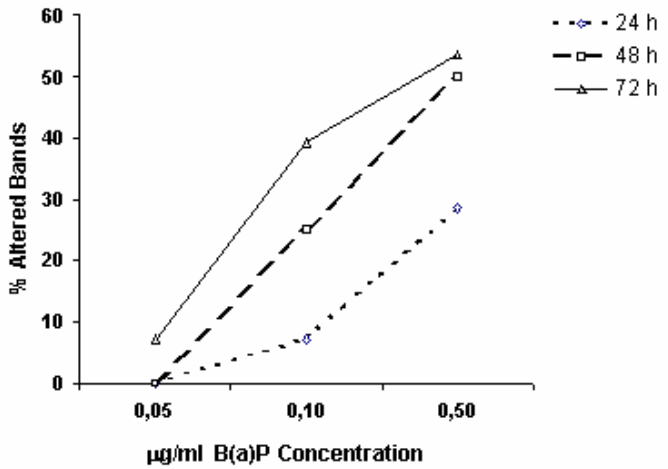
60
50
40
30
20
10
0
% Altered Bands
0,05
0,10
0,50
µg/ml B(a)P Concentration
24 h
48 h
72 h

Figure 2

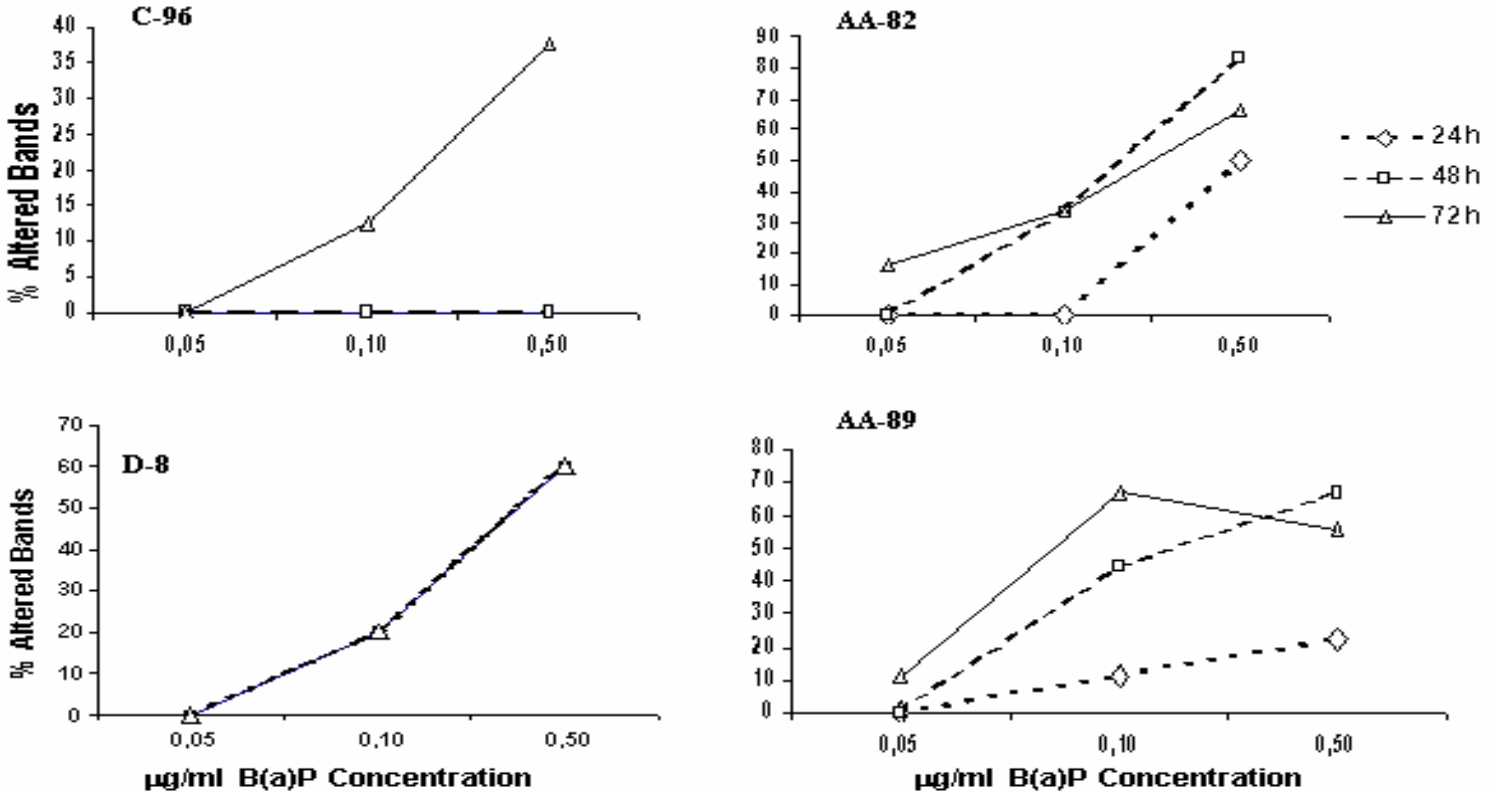
C-96
AA-82
D-8
AA-89
% Altered Bands
µg/ml B(a)P Concentration
0,05
0,10
0,50
24h
48h
72h

Figure 3

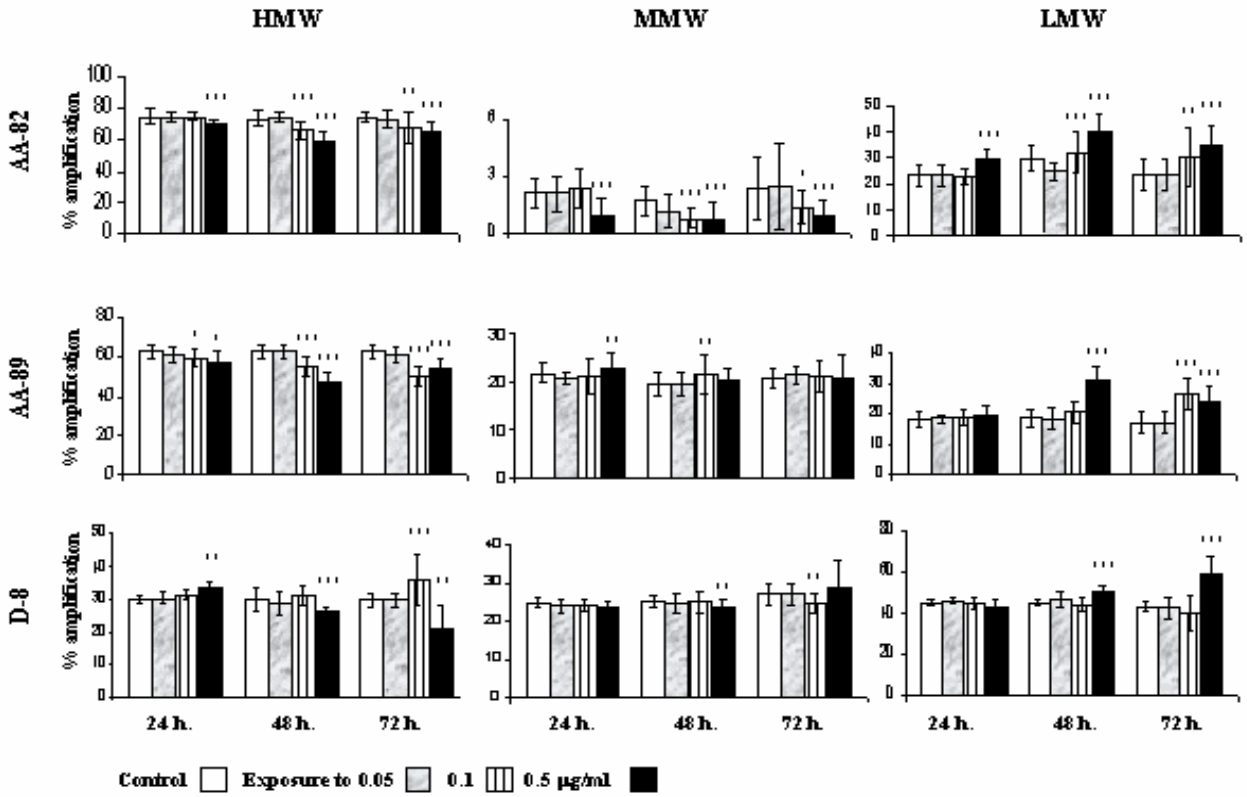
HMW
MMW
LMW
AA-82
AA-89
D-8
% amplification
24 h.
48 h.
72 h.
Control
Exposure to 0.05
0.1
0.5 µg/ml

Figure 4

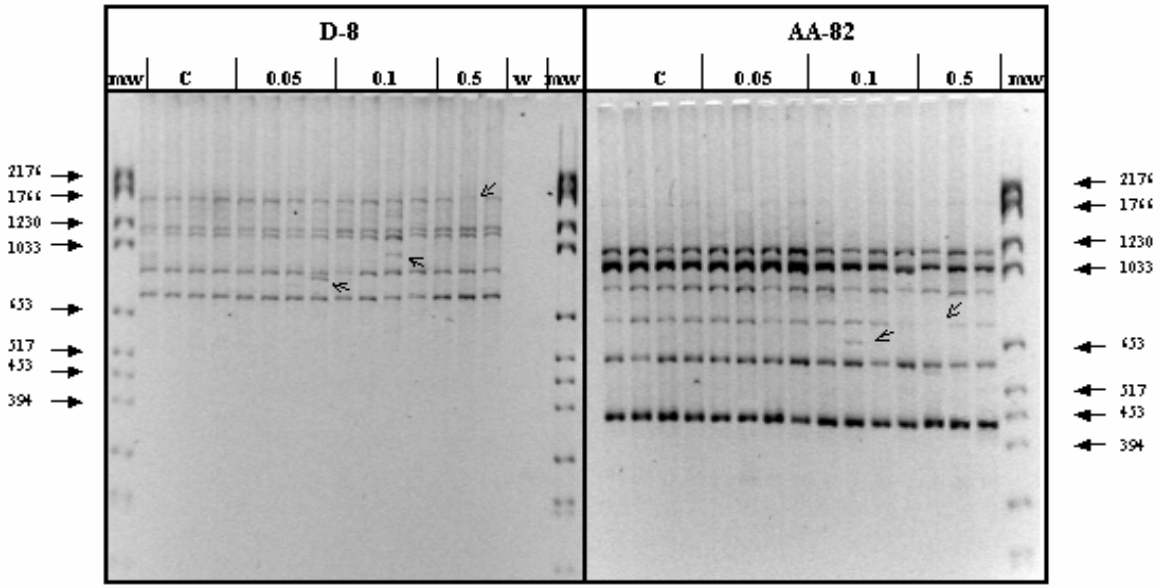
D-8
AA-82
mw
C
0.05
0.1
0.5
w
mw
C
0.05
0.1
0.5
mw
2176
1766
1230
1033
653
517
453
394

Figure 5

ANEXO 4

PUBLICACIONES (en preparación)

ANEXO 4.1

DETECCIÓN DE ALTERACIONES GENETICAS EN TRUCHAS ARCOIRIS EXPUESTAS A BENZO(a)PIRENO

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DETECCIÓN DE ALTERACIONES GENETICAS EN TRUCHAS ARCOIRIS EXPUESTAS A BENZO(a)PIRENO

1. INTRODUCCIÓN

En ecotoxicología la aplicación de líneas celulares derivadas de especies piscícolas, está centrada fundamentalmente en estudios de toxicidad aguda. La línea celular RTG-2 derivada de trucha arcoiris, es una de las mas utilizadas, habiéndose demostrado en estudios de citotoxicidad, una gran capacidad para predecir la toxicidad relativa de sustancias químicas y muestras ambientales (Castaño y col., 1996; Segner, 1998, Braunbeck 2000, Fent 2001) es decir, presenta una muy buena correlación *in vivo* / *in vitro*. Esta línea celular también ha sido aplicada en diversos estudios de genotoxicidad, valorando aberraciones cromosómicas, incremento de micronúcleos, rotura de cadenas etc (Kocan y col., 1982, 1985; Babich y Borenfreund,1995; Kohlpott, y col., 1997; Sánchez y col., 2000; Braunbeck, y col., 2000). En trabajos anteriores

nuestro equipo de investigación ha aplicado la técnica de RAPDs para la detección de daño inespecífico al DNA tras exposiciones agudas y crónicas a genotóxicos patrón.

Las líneas celulares establecidas, presentan una dotación genómica homogénea lo que permite, mediante la técnica RAPD, la obtención de una huella genética o patrón de bandas constante y especifico de su DNA, sin la distorsión que presenta la existencia de bandas polimórficas en la especie in vivo. Esto junto con la posibilidad de evaluar los efectos transgeneracionales que pueden producir las exposiciones crónicas a contaminantes medioambientales, las convierten en un sistema muy adecuado para los estudios de genotoxicidad. No obstante la idoneidad de su aplicación esta en función básicamente, de la constatación de que los resultados obtenidos in vitro sean semejantes a los resultados que se obtengan en estudios in vivo.

En el presente trabajo se realizo un estudio de detección de alteraciones en trucha arcoiris tras una exposición intraperitoneal subletal a Benzo(a)Pireno, el mismo contaminante ambiental utilizado en los estudios in vitro.

Con objeto de valorar los efectos que produce la exposición al toxico en un mismo individuo a lo largo del tiempo, se utilizaron células de sangre periférica que no implicaban el sacrificio del animal.

2. MATERIAL Y MÉTODOS

Se utilizaron un total de 18 truchas de las cuales y por diferentes razones experimentales, solo 8 terminaron el periodo de estudio de 3 meses de

duración. Las truchas, obtenidas de varias piscifactorías, fueron aclimatadas durante un mes antes de la exposición, que se realizó en acuarios individuales de cristal de 50 L con flujo continuo. Los parámetros de calidad del agua se controlaron diariamente (pH, oxígeno disuelto y temperatura) y en el momento de la exposición, el peso medio de los individuos era de aproximadamente 150 grs.

Se realizo una exposición subletal en la mitad de los individuos (T5 a T8) de 69 µg/g de peso corporal de Benzo(a)Pyrene (B(a)P, CAS nº 50-32-8, Sigma, USA) mediante una única inyección intraperitoneal, utilizando aceite de maíz como solvente. Las truchas restantes, (T1 a T4) fueron tratadas con la misma cantidad de aceite de maíz y se utilizaron como control.

Para realizar los análisis, se extrajo 100 µl de sangre periférica de la arteria caudal, inmediatamente antes de iniciar el tratamiento (t_0) y posteriormente, a intervalos de 30 días (t_1, t_2, t_3). Dicha sangre fue recogida en tubos de heparinizados y centrifuga a continuación a 1500 rpm durante 2 min. El sedimento obtenido se resuspendió en buffer citrato (pH 7.4) y se congelaron a –70 ºC en alícuotas de 100 µl hasta su posterior análisis.

2.1. Extracción del DNA y reacción de RAPD

La extracción y amplificación del DNA se realizo mediante el procedimiento optimizado ya descrito en trabajos anteriores. (Ferrero y col., 1998; Becerril y col., 1999)

Las amplificaciones se realizaron utilizando 4 primers (AA-82, AA-89, C-96 y D-8) que previamente habían sido seleccionados por su buena correlación in vivo / in vitro (Becerril y col.,2000). Debido a la presencia de polimorfismos

detectados en los individuos antes de la exposición, se realizo un estudio individualizado de todos los individuos (tratados y no tratados). Para facilitar este estudio, en cada gel se corrieron conjuntamente las reacciones de amplificación realizadas con un solo primer y el DNA procedente de un solo individuo, a los diferentes tiempos (cada uno de ellos por cuadruplicado), un blanco (sin DNA molde) y un marcador de peso molecular (marker IV, Roche, Alemania). Los geles se realizaron por duplicado en diferentes días.

Los productos de amplificación se resolvieron mediante electroforesis en geles de agarosa (2,1% a 140 V durante 4 h) y teñidos con bromuro de etídio. La imagen obtenida en cada gel fue captada utilizando el programa Grab-it (UVP, USA) y, para evitar la subjetividad del operador, fue analizada mediante el programa GelWork 1D (UVP, USA). Las bandas fueron codificadas siguiendo el sistema descrito por Theodorakis y Shugart (1997).

2.2. Análisis de los resultados.

Se llevo a cabo un estudio horizontal intrasujeto, comparando cualitativa y cuantitativamente los patrones de bandas obtenidos a los diferentes tiempos de extracción (t_0, t_1, t_2, t_3,), considerando como control en todos los casos, el perfil fluorimétrico correspondiente a t_0.

El análisis cualitativo consistió en la detección de pérdida o ganancia de nuevas bandas en cada uno de los individuos.

El estudio cuantitativo se realiza comparando estadísticamente los valores del porcentaje de amplificación de las bandas considerando, tanto los valores individualizados, como los valores agrupados en función de sus pesos moleculares. (Becerril y col., 1999)

El análisis estadístico de los resultados se llevo a cabo comparando en primer lugar, la homogeneidad de las varianzas mediante la prueba de Friedman, de cada individuo a lo largo del periodo de estudio y posteriormente, mediante la *t*-Student, las diferencias entre cada uno de los valores t_1, t_2, t_3 y su correspondiente control t_0. El análisis estadístico se realizó mediante el programa SPSS.

3. RESULTADOS y DISCUSIÓN

La presencia de bandas polimórficas, como consecuencia de la variabilidad que presentan los individuos dentro de las poblaciones piscícolas, dificulta la comparación de los patrones de bandas entre diferentes individuos, siendo ésta la mayor limitación que presenta la técnica de RAPD en los estudios *in vivo*. En nuestro caso todos los primers utilizados en este estudio presentaron bandas polimórficas (Figura 1). Por esta razón, se ha realizado un estudio horizontal a lo largo del tiempo para cada uno de los individuos, tratados y no tratados, comparando cualitativa y cuantitativamente, los patrones de bandas obtenidos con cada uno de los primers antes (t_0) y después (t_1 t_2 t_3) de la exposición a B(a)P.

En el análisis cuantitativo, mediante el prueba de Friedman, se advirtieron diferencias significativas en prácticamente la totalidad de las bandas en todos los individuos tratados mientras que en los no tratados ninguna de ellas se mostró alterada (Tabla I). Cuando se estudió la evolución de las alteraciones en función del tiempo para cada individuo, se observó que en los

tratados, el porcentaje de bandas alteradas es máximo en t_2 disminuyendo prácticamente a niveles basales al final del periodo estudiado (t_3) (Figura 2). Esto se justifica por el propio sistema dinámico de renovación de células sanguíneas favorecida, además, por el propio diseño del experimento, ya que los sangrados secuenciales estimulan la eritropoyesis.

Como ya se ha demostrado en trabajos anteriores en los estudios in vitro, las diferencias cuantitativas entre los patrones de bandas quedan mas patentes cuando éstas se agrupan en función de sus pesos moleculares (Tabla ll a y b). No obstante, y por razones obvias, los resultados de dicha agrupación varían en función del primer, es decir, primers con un mayor numero de bandas y distribuidas en un amplio rango de pesos moleculares p.ej. AA-89, AA-82 y C-96, resultan más adecuados que aquellos que amplifican pocas bandas o están desigualmente distribuidas, por ejemplo el D-8. (Figura 1).

En general las bandas de bajo peso molecular presentan en los individuos tratados niveles mas altos de amplificación en detrimento de las bandas de alto peso molecular, confirmando los resultados previamente obtenidos en los estudios in vitro (Figura 3).

Estas alteraciones son consecuencia de cambios inducidos por el toxico en la molécula de ADN. La formación de aductos no solamente inducir cambios estructurales en el ADN sino que reducir la polimerización del ADN y / o bloquear la actividad de la polimerasa. (Atienzar et al 2001).

También en el análisis cualitativo el patrón de bandas de los individuos no tratados permaneció constante a lo largo del periodo de estudio. Sin embargo, en la mitad de los individuos tratados apareció una nueva banda (AA-89_{631}) a partir de t_1 o t_2, (en función del individuo estudiado) permaneciendo

hasta el final del estudio, confirmando los resultados observados *in vitro* tanto en las exposiciones agudas como en las exposiciones a largo plazo. La frecuencia de aparición de dicha banda fue del 100% en todos los casos, confirmando de nuevo lo observado en el caso de la exposición a largo plazo de las células RTG-2 (Fig. 4). Sin embargo, y a diferencia de lo observado en las exposiciones in vitro, no hubo desaparición de bandas.

La aparición de una nueva banda en dos de los individuos, como ya ocurriera en los estudios in vitro, sugiere la presencia de cambios o mutaciones en la secuencia genómica producidos por la acción especifica del B(a)P, como ya se ha observado, utilizando esta misma metodología, en estudios de poblaciones de peces y mamíferos sometidos a otros contaminantes. (Theodorakis y Shugart 1997; Theodorakis y col., 1998, 2000; Shugart y col., 1996; Sugg y col., 1996; Krane y col., 1998; Bickhman y col., 2000). La presencia de cambios en el genoma también justificaría que a los diferentes periodos de tiempo estudiados dicha banda permanezca constante.

Sin embargo, y debido al pequeño número de individuos analizados en este estudio, no es posible extraer conclusiones determinantes ya que la propia susceptibilidad individual requiere el análisis de muestras de gran tamaño. De cualquier manera los resultados obtenidos son, sin duda, la manifestación de las alteraciones producidas por este compuesto en el material genético tanto in vivo como in vitro, demostrando claramente la capacidad de la línea celular RTG-2 tanto para metabolizar este conocido promutágeno, como para predecir los efectos genotóxicos que se observarían en los individuos expuestos. Es evidente que para poder transferir dichos resultados in vitro a una escala

poblacional son necesarios estudios más completos y ambiciosos que exceden los objetivos de este trabajo.

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Tabla 1. Análisis cuantitativo: prueba de Friedman para el análisis de varianzas del total de bandas amplificadas en los periodos de muestreo (t_0, t_1, t_2, t_3) para cada uno de los individuo, tratados (B) y no tratados (A).

	A				B			
Bandas	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**	**T7**	**T8**
C-96$_{1714}$	**ns**	**ns**	**ns**	**ns**	**b**	**c**	**b**	**b**
D-8$_{1554}$	**ns**	**ns**	**ns**	**ns**	**ns**	**c**	**c**	**a**
C-96$_{1321}$	**ns**	**ns**	**ns**	**ns**	**b**	**b**	**c**	**b**
D-8$_{1172}$	**ns**	**ns**	**ns**	**ns**	**c**	**a**	**c**	**ns**
D-8$_{1112}$	**ns**	**ns**	**ns**	**ns**	**ns**	**b**	**c**	**ns**
C-96$_{1099}$	**ns**	**ns**	**ns**	**ns**	**b**	**ns**	**------**	**ns**
AA-82$_{1075}$	**ns**	**ns**	**ns**	**ns**	**b**	**ns**	**b**	**a**
AA-82$_{965}$	**ns**	**ns**	**ns**	**ns**	**c**	**ns**	**c**	**a**
C-96$_{965}$	**-----**	**------**	**------**	**------**	**ns**	**a**	**------**	**a**
C-96$_{856}$	**ns**	**ns**	**ns**	**ns**	**ns**	**c**	**ns**	**ns**
AA-82$_{840}$	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**
D-8$_{826}$	**ns**	**ns**	**ns**	**ns**	**c**	**b**	**ns**	**ns**
AA-82$_{700}$	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**	**a**	**a**
D-8$_{686}$	**-----**	**-----**	**ns**	**ns**	**-----**	**-----**	**-----**	**-----**
C-96$_{642}$	**ns**	**ns**	**ns**	**ns**	**c**	**a**	**ns**	**ns**
AA-82$_{640}$	**-----**	**-----**	**-----**	**-----**	**-----**	**ns**	**-----**	**ns**
C-96$_{598}$	**ns**	**-----**	**-----**	**-----**	**ns**	**-----**	**ns**	**-----**
AA-82$_{570}$	**-----**	**ns**	**ns**	**ns**	**a**	**a**	**b**	**a**
C-96$_{487}$	**ns**	**ns**	**ns**	**ns**	**c**	**a**	**c**	**a**
AA-82$_{430}$	**ns**	**ns**	**ns**	**ns**	**b**	**b**		**b**
C-96$_{380}$	**ns**	**ns**	**ns**	**ns**	**c**	**c**	**c**	**a**

ns = diferencias no significativas frente al control.

Diferencias significativas frente al control a = *P*<0,05; b = *P*<0,005: c = *P*<0,001

Numero de replicados = 8

Tabla 2. Análisis cuantitativo comparando los porcentajes de amplificación individualizados de cada una de las bandas y agrupado según sus pesos moleculares, antes y después de la exposición a B(a)P.

	t_0-t_1				t_0-t_2				t_0-t_3			
Bandas	**T5**	**T6**	**T7**	**T8**	**T5**	**T6**	**T7**	**T8**	**T5**	**T6**	**T7**	**T8**
AA-82$_{1075}$	b	ns	a	ns	b	ns	ns	ns	ns	ns	ns	ns
AA-82$_{965}$	c	a	c	ns	c	a	a	b	b	ns	ns	ns
AA-82$_{840}$	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Alto$_{pm}$	c	b	c	c	c	b	c	c	ns	ns	c	ns
AA-82$_{700}$	ns	ns	ns	b	ns	ns	a	a	ns	ns	ns	ns
AA-82$_{640}$	------	ns	------	ns	------	ns	------	ns	------	ns	------	ns
Medio$_{pm}$												
AA-82$_{570}$	ns	ns	c	ns	c	a	ns	b	a	ns	ns	ns
AA-82$_{430}$	c	ns	b	ns	c	b	a	b	ns	b	ns	ns
Bajo$_{pm}$	b	a	c	c	c	b	b	c	ns	ns	b	ns
C-96$_{1714}$	b	a	b	b	a	ns	b	b	ns	ns	ns	ns
C-96$_{1321}$	a	b	c	a	ns	c	b	ns	ns	ns	ns	ns
C-96$_{1099}$	b	a	------	ns	ns	a	------	ns	ns	ns	------	ns
Alto$_{pm}$	b	b	b	ns	ns	ns	b	ns	ns	ns	ns	ns
C-96$_{964}$	ns	a	------	ns	ns	b	------	b	ns	a	------	b
C-96$_{856}$	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Medio$_{pm}$	a	a	a	ns	a	ns	ns	ns	ns	ns	ns	ns
C-96$_{642}$	b	b	ns	ns	c	ns	a	ns	a	ns	ns	ns
C-96$_{598}$	ns	------	ns	------	ns	------	ns	------	ns	------	ns	------
C-96$_{487}$	a	b	b	a	c	a	a	a	b	ns	ns	ns
C-96$_{380}$	c	c	a	b	a	ns	c	a	ns	ns	ns	ns
Bajo$_{pm}$	c	a	b	ns	ns	a	ns	ns	ns	ns	ns	ns
D-8$_{1554}$	ns	ns	a	ns	ns	c	b	b	ns	ns	ns	ns
Alto$_{pm}$												
D-8$_{1172}$	b	a	c	ns	c	ns	ns	ns	b	ns	ns	ns
D-8$_{1112}$	ns	a	c	ns	b	a	ns	ns	ns	a	ns	ns
Medio$_{pm}$	c	a	a	ns	b	a	c	b	ns	ns	ns	ns
D-8$_{826}$	c	a	ns	ns	c	ns	ns	ns	a	ns	ns	ns
Bajo$_{pm}$												
AA-89												
Alto$_{pm}$	c	b	c	c	c	b	c	c	ns	ns	c	ns
Medio$_{pm}$	ns	c	a	ns	ns	c	a	ns	ns	ns	b	ns
Bajo$_{pm}$	b	a	c	c	c	b	b	c	ns	ns	b	ns

ns = diferencias no significativas frente al control. t_0 = antes de la exposición t_1, t_2 y t_3 = 1, 2 y 3 meses después de la exposición.
Diferencias significativas frente al control a = *P*<0,05; b = *P*<0,005: c = *P*<0,001
Numero de replicados = 8

Figura 1. Perfiles de amplificación obtenido para cada uno de los primers mostrando la presencia de bandas polimórficas (►). Las bandas de cada uno de ellos se agruparon en a: alto, b: medio y c: bajo peso molecular.

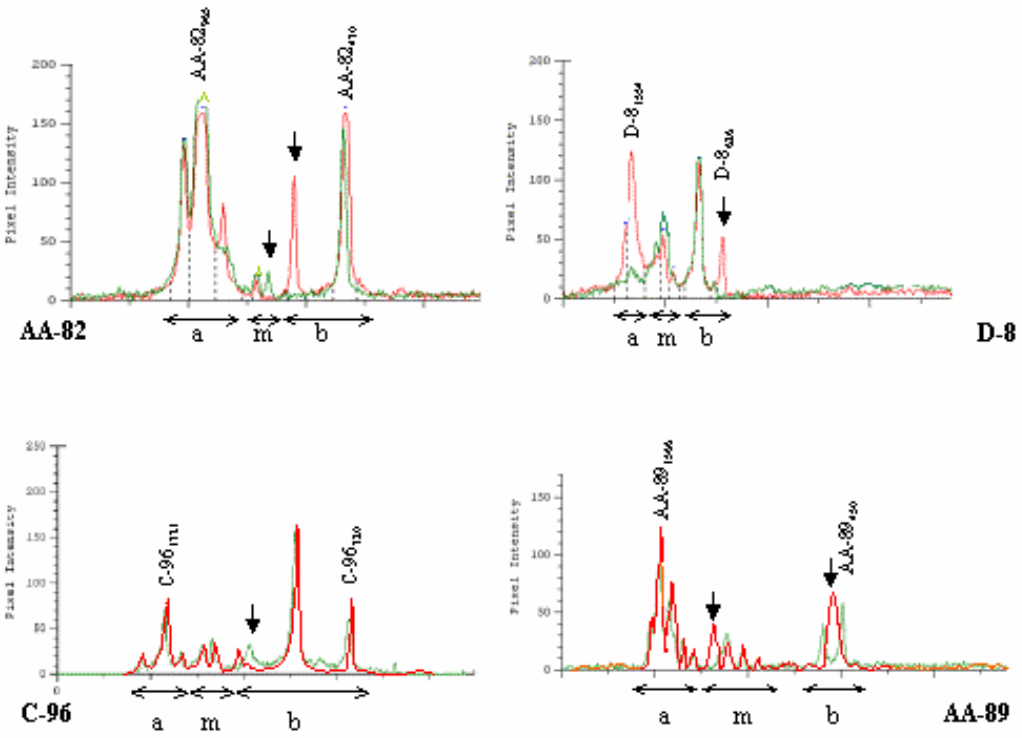

Figura 2. Análisis cuantitativo: porcentaje total de bandas alteradas en individuos tratados cada uno de los periodos analizados.

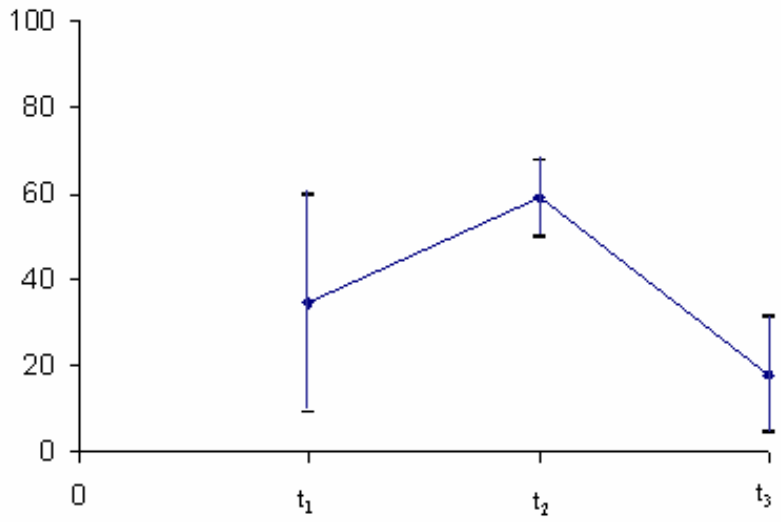

Figura 3. Representación grafica mediante diagrama de cajas de los porcentajes de amplificación agrupados en alto medio y bajo peso molecular para cada primer. En los individuos tratados (B) se observa en general una perdida de amplificación en las bandas de alto peso molecular en función de las bandas de bajo peso molecular. Los individuos no tratados (A) no muestran alteraciones observables significativas en ninguno de los primers

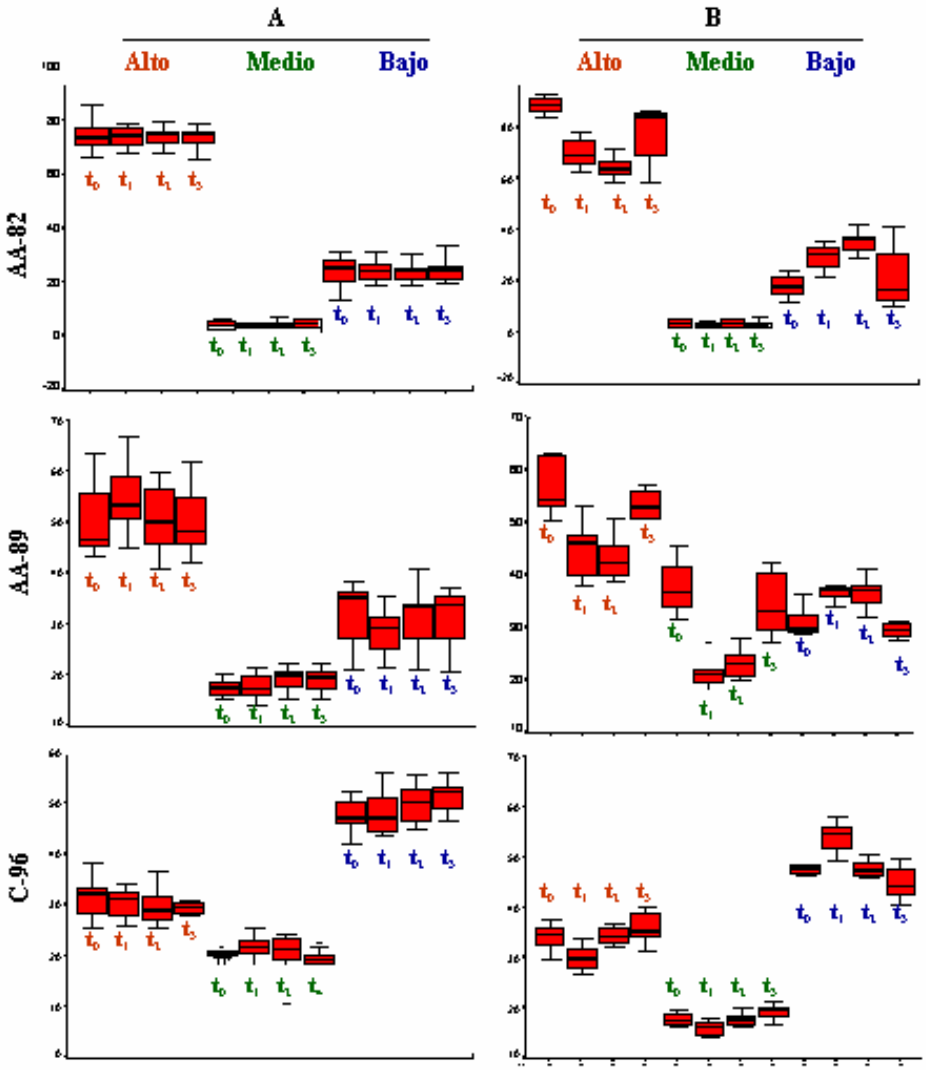

Figura 4. Patrón de bandas del primer AA-89 obtenido a partir de individuos tratados (B) y no tratados (A) con B(a)P. En los tratados se observa la aparición de una nueva banda (→) a partir de t_1 en el 100% de las amplificaciones hasta el final del periodo estudiado. En ellos se muestra la aparición de bandas polimórficas (}-).
m: marcador de peso molecular en pares de bases. b: blando