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Conventional armament wastes induce micronuclei in wild brown trout *Salmo trutta*

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Abstract

We analysed micronuclei in brown trout *Salmo trutta* specimens sampled in the Trubia River, upstream and downstream of the emissions from a Spanish military factory to assess genotoxicity risks derived from military wastes. A significant exponential increase in micronuclei counts was found in fish living downstream of the military wastes with respect to fishes inhabiting upstream areas of the same river. In comparison, we only found a linear increase in micronuclei counts in a control stream where an old military factory had been demolished 6 months before sampling. This difference suggests that active discharge of armament factory wastes can directly induce micronuclei and therefore represents a genotoxic risk for the ecosystem.

Keywords: Micronuclei; Trout; Armament wastes; Genotoxicity

1. Introduction

Factories and facilities that construct and test non-nuclear weapons usually produce wastes that include a large number of hazardous agents such as heavy metals. The problem of armament wastes is especially severe in cases of old armament factories, where facilities for recycling disposal or storing dangerous chemicals are obsolete or absent. An example in Spain is the Santa Barbara Company (Fabrica de Armas), dedicated to the fabrication of conventional armament, whose effluents empty into the Trubia River, a tributary of the Narcea-Nalon watershed (Asturias, North Spain). Three emittors directly discharge into the river along a one kilometer stretch. In this river, wild fish populations of different species are exploited by anglers for human consumption (e.g. brown trout *Salmo trutta*, Atlantic salmon *Salmo salar*, European eel *Anguilla anguilla*). In the same watershed, the River Gafo, a stream of similar ecological conditions, received wastes of an obsolete enterprise related with military industry for more than two decades, but the factory was demolished in early 1999. Some problems of land contamination with mercury were reported, but water pollution has not been detected. At both these locations it is important to assess the effects of current discharge and remaining pollution on fish populations.

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Freshwater fish species such as brown trout and European eel can bioaccumulate heavy metals, constituting a health risk for consumers. In some north Spanish river systems, high levels of heavy metal pollution have been found in 38% of fish of both species that were potential catches for sport fishermen [1]. Brown trout is the most abundant and dominant species of Asturian freshwater ecosystems; thus, it has been used as a subject of several short and long term genotoxicity assays [2] and also as a biomonitor of heavy metal pollution [3,4].

Micronuclei are small fragments of chromatin separated from the main cell nucleus which are evidence of chromosome breaking or mitotic spindle dysfunction and are frequently produced by genotoxic agents [5]. Micronuclei are produced in all cell types after an irregular division process in which a chromosome fragment or a whole chromosome is not lost during the anaphase but is delayed with respect to the rest of chromosomes, constituting a small secondary nucleus in interphase [6]. Since the development of the test [7], micronuclei counts are considered good short-term indicators of cytogenetic damage because they are simple, sensitive and independent of particular karyotype characteristics [5]. Although originally developed for its application in mouse, it was subsequently modified by Hooftman and de Raat [8] for the application in the laboratory to fish. It is widely employed to assess the genotoxic effects of a wide range of chemical compounds under direct or indirect exposure in vivo [9–11,12]. In environmental mutagenesis investigations, the micronucleus test in fish has served as a simple, reliable, sensitive and inexpensive procedure to assess the biological impact of freshwater pollution [2,13–15].

The aim of this work is to assess possible risks for freshwater ecosystems (and eventually for human as fish consumers) derived from the non-nuclear armament wastes cited above, using the micronucleus test in brown trout as an in situ indicator of genotoxicity.

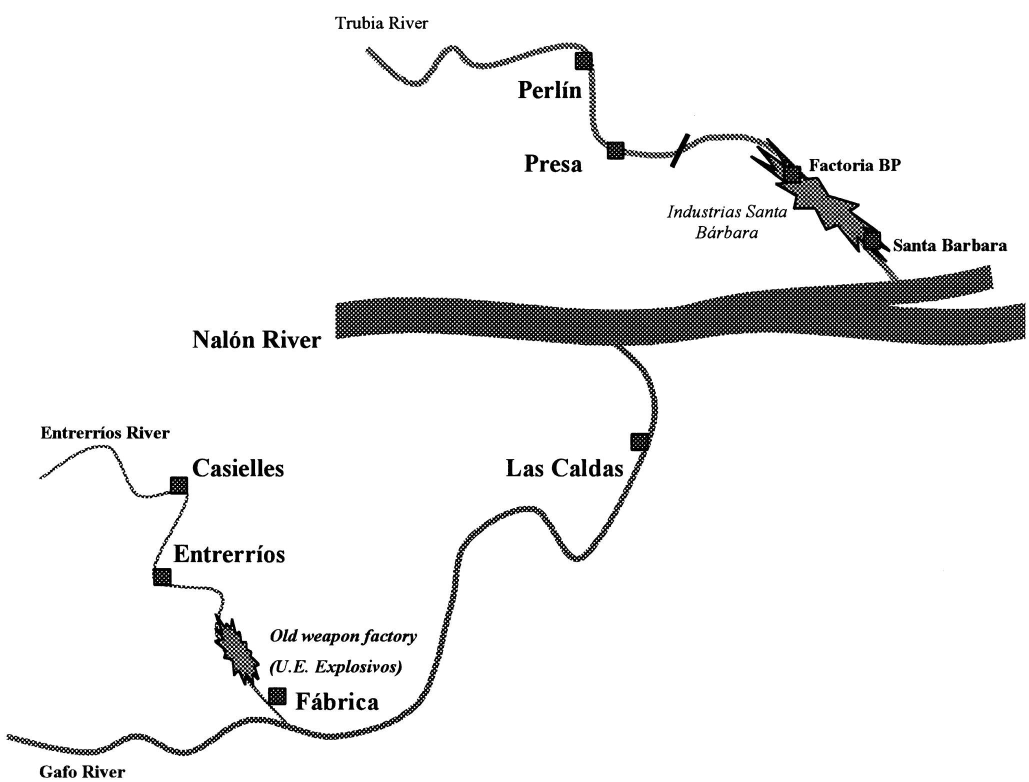

Fig. 1. Sampling sites located in both Trubia and Gafo rivers (grey squares). Armament wastes discharge areas are denoted as grey stars.

We compared micronuclei counts of specimens caught upstream and downstream from the factory wastes in both Trubia and Gafo rivers.

2. Material and methods

In July 1999, 1-year-old brown trout juveniles (sedentary, feeding in reduced areas of the benthic community; see [16]) were caught by electrofishing in the river areas showed in Fig. 1. Table 1 presents water environmental parameters analysed by the authorities in charge of water management (Confederacion Hidrográfica del Norte de España and Principado de Asturias: Consejería de Medio Ambiente, official records 1999) in the areas affected by the armament wastes, and Table 2 presents heavy metal contents in sediments in three of the sampled areas in the Trubia River. Of note are the high metal contents in the lowest area sampled (Santa Barbara). Only 1-year-old brown trout individuals were considered for micronuclei analysis. They were transported alive to the laboratory, then sacrificed adding the anaesthetic Ethyleneglycolmonophenylether (Merck Cat. No 8.07291.1000) to water in a small receptacle (20 ml/l) to avoid animal suffering. Length and weight of each fish were recorded and the condition factor was calculated as follows: $W \times 100/L^3$, where W is total fish weight in grams and L represents fish length in centimeters. After fish sacrifice, a small amount of cephalic kidney was removed from each specimen and spread onto clean slides. The slides were air-dried for 10 min, then stained.

Table 1
Characteristics of rivers sampled[a]

Characteristics	Gafo River[b]	Trubia River[c]
pH	7.3	8.19
EC (μS/cm)	581	391
NO_3^- (mg/l)	22.59[d]	3.15[d]
P (mg/l)	<0.025	0.30[d]
Ca (mg/l)	96.63	64.5
Mg (mg/l)	4.27	7.2
K (mg/l)	1.47	1.7
Al (mg/l)	0.048	–
Sr (mg/l)	0.07	–
Cu (mg/l)	<0.01[d]	<0.01[d]
Pb (mg/l)	<0.01	<0.005
Zn (mg/l)	0.01[d]	<0.01
Ni (mg/l)	<0.01	<0.06
Mn (mg/l)	<0.01	0.05
Fe (mg/l)	<0.05	0.21
Cr (mg/l)	<0.01	<0.02
Cd (mg/l)	<0.005	0.0005
Hg (mg/l)	<0.001	<0.001

[a] Water contents (expressed in mg/ml) of several elements analysed by the authorities in charge of water management.
[b] Principado de Asturias. Consejería de Medio Ambiente.
[c] Confederación Hidrográfica del Norte. Official records September 1999.
[d] Indicate contents overcoming legal limits (CEE/86/280). EC: Electric conductivity at 20°C.

Table 2
Characteristics of rivers sampled[a]

Samplig site	Cu (ppm)	Zn (ppm)	Cd (ppm)	Pb (ppm)
Presa	18.5	77	0.12	29.5
Factoria Bp	39.9	127	0.22	48.5
Santa Barbara	480	6737	3.37	104

[a] Metal contents in sediments (<2 mm) expressed as mg/g dry weight in several sampling sites of the Trubia River (source: Universidad de Oviedo. Dpto. Quimica Analitica).

Samples were sequentially stained with May-Grünwald for 2 min, May-Grünwald/distilled water 1:1 for 3 min and Giemsa/distilled water 1:6 for 10 min. Then slides were rinsed with distilled water, allowed to dry and mounted with Eukitt resin. From each animal, 1000 renal erythrocytes were scored under 1000× magnification to determine the frequency of micronuclei. To be considered as micronuclei, particles were non-refractory, with the same colour as the cell nucleus, round or ovoid shaped, with a diameter 1/5–1/20 of the erythrocytes and completely separated of the main nucleus.

Statistical analyses included contingency chi-square tests and regression analyses based on b and r values, and ANOVA tests of fitness of data to the regression shape [17]. They were performed using the statistical package SPSS version 8.0 for PC.

3. Results

Table 3 presents condition factor and micronuclei counts for the Trubia and Gafo rivers. In both situations, we found a significant increase of micronuclei averages in fish caught in downstream locations with respect to upstream areas. Likewise, a decrease in the condition factor of the individuals sampled along

Table 3
Biological Characteristics and micronuclei frequencies for the Trubia and Gafo rivers[a]

Sampling site	N	CF ± S.D.	Micronuclei ± S.D.
Gafo River			
Casielles	14	1.339 ± 0.428	1.428 ± 1.395
Entrerríos	15	1.227 ± 0.442	1.866 ± 1.726
Fábrica	10	1.212 ± 0.125	2 ± 1.699
Las Caldas	24	1.180 ± 0.143	2.375 ± 1.906
Trubia River			
Perlín	10	1.403 ± 1.198	0.4 ± 0.699
Presa	17	1.35600 ± 0.194	0.705 ± 0.771
Factoría BP	7	1.285 ± 0.078	1.571 ± 1.511
Santa Barbara	10	1.238 ± 0.127	4.1 ± 1.370

[a] Condition factor (CF) and micronuclei averages per 1000 erythrocytes and standard deviations (S.D.) in fish sampled in both Gafo and Trubia rivers at different sampling sites.

the water course was observed in both rivers. In the River Gafo, micronuclei counts ranged from 1.4 to 2.4, increasing downstream. They statistically fitted a linear regression ($F = 55.86$, $P < 0.05$), as shown in Fig. 2 ($y = 1.173x + 0.297$, significantly different from $b = 0$ with $P < 0.05$). Brown trout condition factor, although decreasing downstream (Fig. 3) did not fit a linear regression ($F = 10.76$, n.s.); the decrease was not statistically significant ($b = -0.0492$, $r = 0.9183$, n.s.).

In the River Trubia, micronuclei counts in upstream locations (Perlin: 0.4; Presa: 0.7 per 1000 erythrocytes) were lower that those found for the River Gafo. In the area affected by the armament emissions, they increased considerably, specially in the last sampling site (Santa Barbara). The increase in micronuclei averages along the watercourse was statistically significant. But in this situation, we found clear evidence that there was an exponential increase of micronuclei coincident with the exposure of trouts to wastes, as shown in Fig. 4. Micronuclei frequencies in the four samples statistically fitted an exponential shape ($F = 156.07$, $P < 0.01$), not a linear model. The increase of micronuclei counts in Santa Barbara location was notable (2.5 micronuclei) with respect to Factoria BP, providing a statistically significant difference with respect to the location placed immediately upstream (Factoria BP versus Trubia: $X^2 = 26.63$, $P < 0.001$). For individual condition factor, decreasing downstream, the values observed fitted a linear regression model ($F = 278.09$, $P < 0.01$) as shown in Fig. 5

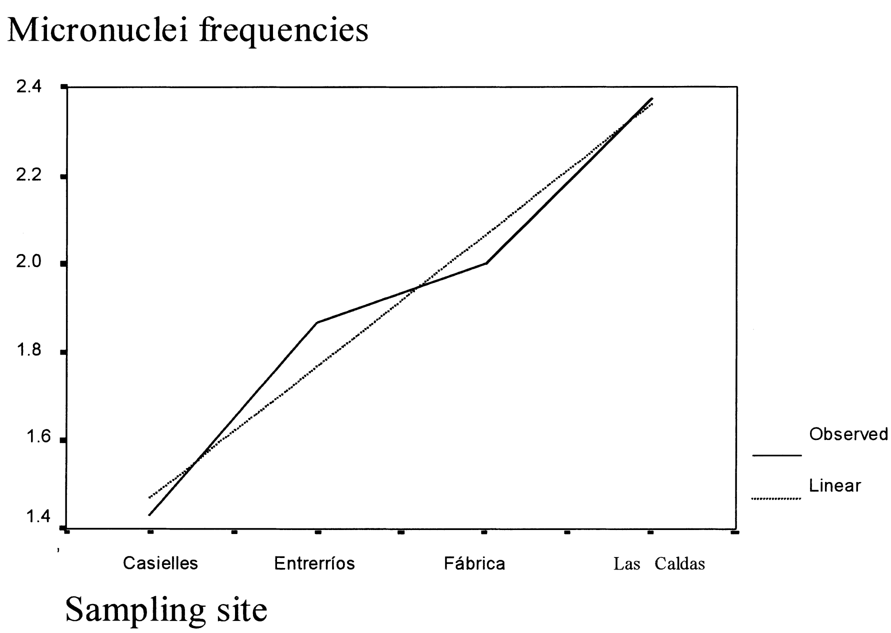

Fig. 2. Mean of micronuclei in brown trout sampled from the River Gafo (observed) and linear regression model obtained from data (linear).

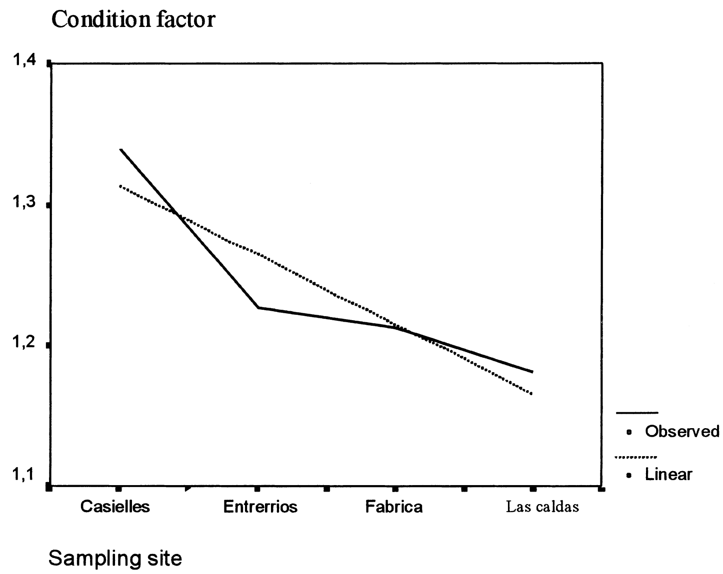

Fig. 3. Condition factor averages in brown trout sampled from River Gafo (observed) and linear regresion model obtained from data (linear).

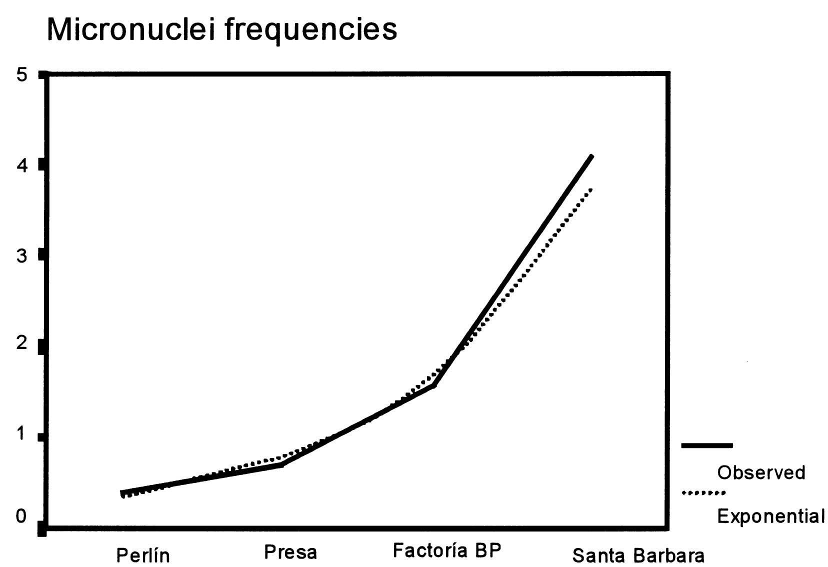

Fig. 4. Mean of micronuclei in brown trout sampled from the River Trubia (observed) and exponential regression (exponential).

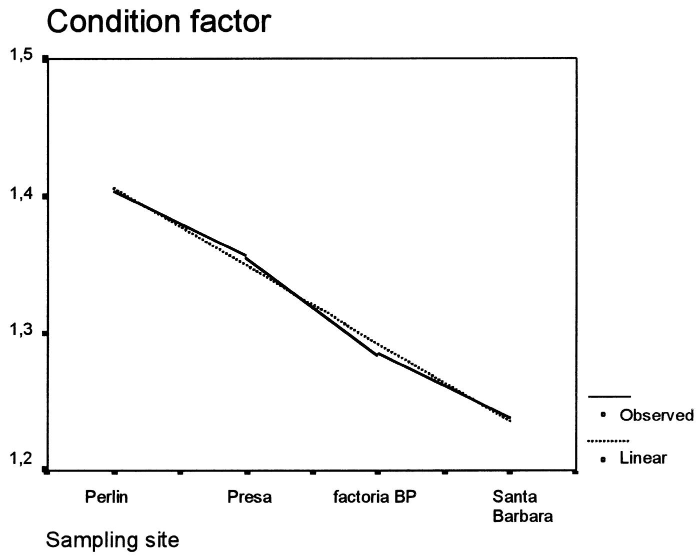

Fig. 5. Condition factor averages in brown trout sampled from River trubia (observed) and linear regresion model obtained from data (linear).

($y = 1.462x - 0.056$, significantly different from $b = 0$ with $P < 0.01$).

4. Discussion

Our results confirm that, as previously reported [2], the micronucleus test is sensitive enough to reveal significant differences between brown trout populations living in ecosystems with different pollution levels. The simple progressive increase of micronuclei frequency along a watercourse found for the River Gafo is a good example that micronuclei counts are associated, in the wild, with pollution levels expected when a gradient of pollution exists along the water course. On the other hand, the range of micronuclei averages found in our samples are similar to those described by other authors for other species, such as rainbow trout [15] and barbel [15] in other polluted sites. In contrast, Sanchez-Galan et al. [2] reported higher micronuclei values similar only to those found in our most polluted site (Santa Barbara) for brown trout inhabiting other Spanish rivers. The main difference existing between the Sanchez-Galan et al. [2] methodology and ours was the type of nuclear abnormalities recorded: they considered lobulated or bilobulated nuclei in addition to micronuclei, whereas we counted only micronuclei. Many different nuclear abnormalities have been reported for fish species [18–20], but there is not a consensus about the type of aberrations to be included in the micronucleus test. There are few data concerning the inclusion of these abnormalities in in situ studies. For example, Carrasco et al. [19] found a lack of correlation between any observed variation of the nuclear morphology and any measured level of chemical contamination in the sediments or in fish tissues. However, micronuclei counts have been reported as a sensitive assay to detect water pollution in field experiments [14,15]. To be conservative, we have chosen to record only micronuclei forms, the nuclear aberration commonly accepted as indicator of genotoxicity in fish. Due to the kinetics of the micronucleus formation, immature erythrocytes can be more sensitive to detect acute effects of chemicals; a specific dye (i.e. acridine orange) can be used to distinguish between immature and mature erythrocytes [21]. Nevertheless, for monitoring purposes the micronucleus test in mature fish erythrocytes using classic DNA staining procedures is sensitive and reliable enough to detect the genotoxic effects of continuous (not acute) chemical pollution in aquatic ecosystems [22], therefore the use of other type of dyes, more specific, is not considered to be relevant for the present work.

The two situations here presented are different. Although they both are theoretically influenced by armament-related wastes, in the River Gafo the increase of micronuclei detected along the watercourse can be interpreted as the normal situation expected for a stream with a gradient of pollution, not to a direct effect of armament wastes. We cannot discard the effect of remaining pollution from the recently demolished factory in increasing micronuclei in trout caught downstream, but we can neither directly relate such an increase with armament wastes. This conclusion is consistent with the results of water analyses for this river (Table 1): no significant quantities of any genotoxic element were detected in the area formerly affected by old wastes.

In the River Trubia, the exponential increase of micronuclei in brown trout can be directly attributed to the discharge of the conventional armament factory wastes. This conclusion is supported by the water quality analyses in the area affected by armament wastes, which indicate a poor water quality (high levels of NO_3^-, P and Cu) (Table 1); in addition, the presence of heavy metals in the river sediments (Table 2) clearly points out the existence of persistent heavy metal pollution associated with the factory, although it was not detected in the water sample analysed. The significant decrease of trout condition factor downstream can also be considered an indirect indicator of a poor habitat status in the lower sampling sites. Feeding habits of young brown trout are predatory, not related to the bottom or to the sediments [23], therefore any remaining product included in sediments does not probably affect directly the specimens sampled in this work. Nevertheless, the possible accumulation of heavy metals in the tissues of small aquatic invertebrates could lead to their accumulation in brown trout tissues. The presence of heavy metals in sediments is likely due to periodic discharges of wastes with high heavy metal content and most water samples have probably been taken when discharges were not occurring. In experimental conditions, heavy metals induce micronuclei expression in brown trout [24], we, therefore, conclude that the high micronuclei frequencies we observed are consistent with a situation of water pollution and that micronuclei were likely induced, at least partially, by the same heavy metal pollution that produced heavy metal deposits in sediments.

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