Eur J Forest Res (2012) 131:1889–1903
DOI 10.1007/s10342-012-0640-7

ORIGINAL PAPER

Assessment of the toxicity of ash-loaded runoff from a recently burnt eucalypt plantation

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Received: 13 July 2011 / Revised: 17 March 2012 / Accepted: 25 May 2012 / Published online: 7 July 2012

Abstract Although wildfires are identified as an important source of polycyclic aromatic hydrocarbons (PAHs) and PAHs are well-known for their pernicious properties, the toxicity of runoff from recently burnt areas has received little research attention. This knowledge gap was addressed here through laboratory assays in which four aquatic species from distinct trophic levels were exposed to different dilutions of ash-loaded runoff. The runoff was collected in a recently burnt eucalypt stand in north-central Portugal on two occasions, immediately after the wildfire and about 1 year later. The total PAH load was about four times higher at the first than second sampling occasion (1194 vs. 352 ng l^{-1}) but even the latter value was considerably higher than those reported by prior studies on burnt areas. In addition, the two runoff samples differed noticeably in PAH composition, with a clear predominance of naphthalene in the second sample. Both runoff samples produced significant inhibitory effects on the three species representing the lower trophic levels, that is, the bacteria *Vibrio fischeri*, the algae *Pseudokirchneriella subcapitata* and the macrophyte *Lemna minor*. The invertebrate *Daphnia magna* was not significantly affected but chronic tests are needed to discard the probable propagation of toxic effects from the lower trophic levels. Surprisingly, the runoff collected 1 year after the wildfire was the most toxic to *V. fischeri*, *P. subcapitata* and *L. minor*. Possibly, this was due to predominance of naphthalene in this sample. Surely, however, this demonstrated that detrimental off-site effects of wildfires are not necessarily limited to the immediate post-fire situation.

Keywords Wildfire impacts · Ecotoxicological effects · Polycyclic aromatic hydrocarbons (PAHs) · Ash-loaded runoff · Eucalypt · Aquatic species

Communicated by A. Merino.

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Introduction

Although wildfires are now widely recognized as a natural phenomenon in Mediterranean regions, present-day fire regimes strongly reflect human activities, in particular the widespread planting of highly flammable tree species in combination with land abandonment, resulting amongst others in an increase in fuel load (e.g. Lloret 2004; Moreira et al. 2009; Carmo et al. 2011; Shakesby 2011). In Portugal, wildfires are particularly problematic since have been affecting large areas in the past three decades, on average some 100,000 ha per year and over 400,000 ha in a dramatic year like 2003 (Pereira et al. 2005). Fire frequency in Portugal is also not expected to decrease in the foreseeable future, not just because of the likely increase in fire-propitious meteorological conditions not only due to climate change but also because of the nature of the country's forestry activities (Pereira et al. 2006; Fischlin et al. 2007).

Wildfires are an important societal and environmental concern worldwide, since their adverse effects are manyfold and include public safety and health, economic damages and costs (through fire prevention and fighting), air and water pollution, land-use sustainability and biodiversity. In the case of surface water pollution—the topic of this study—it is well documented that wildfires, through their direct effects on vegetation cover and soil properties, can lead to considerable changes in hydrological processes and the associated transport of sediments, nutrients and pollutants to downstream aquatic and flood zone ecosystems and man-made constructions (Shakesby and Doerr 2006).

Whilst in general wildfire effects are much better studied with respect to runoff amounts than their physico-chemical composition (Shakesby and Doerr 2006), fire-induced pollution of surface water by polycyclic aromatic hydrocarbons (PAHs) has been overlooked till quite recently (Olivella et al. 2006; Vila-Escalé et al. 2007; Schäfer et al. 2010). In a nutshell, the principal findings of these recent studies were that (1) the export of PAHs from burnt areas into downstream water bodies could be increased substantially (by at least a factor 3); (2) this effect was short-lived, dependent on distance to the burnt area; (3) it did not produce a health risk since observed total PAH concentrations were below the limit for drinking water established by the European Community.

PAHs are pyrolytic substances that are classified as priority pollutants by the United States Environmental Protection Agency (USEPA 2002a) for their well-known toxic, mutagenic and carcinogenic properties as well as their environmental persistence and tendency for bioaccumulation along the food chain (ATDSR 1995; IARC 1998; Boström et al. 2002). PAHs have been found to produce a wide range of ecotoxicological effects in a diverse suite of organism, including microorganisms, terrestrial plants, amphibians, reptiles and mammals (e.g. Wang and Busby 1993; Long et al. 1995; Delistray 1997; Rappaport et al. 2004; Cachot et al. 2006; Hellou et al. 2006). The reported effects involved acute toxicity, negatively affecting survival, growth and/or metabolic activity, as well as developmental and reproductive toxicity, enhancing DNA mutation, cancer formation and acting as endocrine disruptors (Delistray 1997; Clemons et al. 1998; Hellou et al. 2006). In spite of these adverse effects of PAHs are well-established, the role of PAHs seems to have been disregarded in prior studies that showed noticeable consequences of wildfires on aquatic biota, including periphyton, macroinvertebrates and fish (e.g. Earl and Blinn 2003; Minshall 2003; Lyon and O'Connor 2008).

The present study addressed, first and foremost, the lack of information on the toxic effects of runoff from burnt areas. Furthermore, to the best of our knowledge, it is the first assessment of PAH loads of surface water in recently burnt areas in Portugal. Both research gaps are particularly pertinent, now that the EU Water Framework Directive (WFD) is in its implementation phase and the need exists to duly identify diffuse sources of contamination of aquatic systems. The specific objectives of this study were to assess (1) the ecotoxicological effects of runoff from a recently burnt eucalypt plantation in north-central Portugal, both immediately after the wildfire and 1 year later, using four aquatic species from different trophic/functional levels (*Vibrio fischeri*, *Pseudokirchneriella subcapitata*, *Lemna minor* and *Dapnhia magna*); (2) the PAH loads of the runoff collected at the two above-mentioned sampling occasions; (3) the relationships of the ecotoxicological effects on the four species with the observed PAH loads, also addressing the possible confounding role of nutrient availability.

Materials and methods

Study area and site

The study area is located near the hamlet of Colmeal, on the border of the municipalities of Góis and Arganil, in the Coimbra District of north-central Portugal (40° 08′ 46″ N, 7° 59′ 35″ W, 500 m asl; Fig. 1). The area consisted of some 70 ha that were consumed by a wildfire on 24 August 2008, and it was selected to study post-fire on soil erosion in the framework of the EROSFIRE-II project. The climate of the study area is of a transitional Atlantic-Mediterranean type, with wet winters and dry summers. According to the available information (in map format: APA 2011), the mean annual temperature is between 10 and 12.5 °C, whereas the average annual rainfall is between 1,400 and 1,600 mm. The geology of the area is composed of pre-Ordovician schists and greywackes (Ferreira 1978; Pimentel 1994), which have given rise to shallow soils that typically correspond to Humic Cambisoils (Cardoso et al. 1971, 1973). Prior to the wildfire, the study area was predominantly covered by fast-growing eucalypt plantations (Eucalyptus globulus Labill.) for paper pulp production, with the remaining 25 % or so consisting of Maritime Pine stands (*Pinus pinaster*).

Within the burnt area, five hill slopes were each instrumented with an unbounded slope-scale plot for monitoring post-fire erosion from late August 2008 onwards (Fernandes et al. 2010). One of these slopes was selected for the present study as being representative of the area's predominant land cover as well as of the prevalent, moderate-to-low fire severity. Fire severity was surveyed for the burnt area as a whole, by photography and visual inspection, from several look-out points, of the degree of the tree canopy consumption by the wildfire in the various forest stands (see also Maia et al. 2012). This involved distinguishing between light-coloured patches where canopies were scorched only (low severity) and dark-coloured

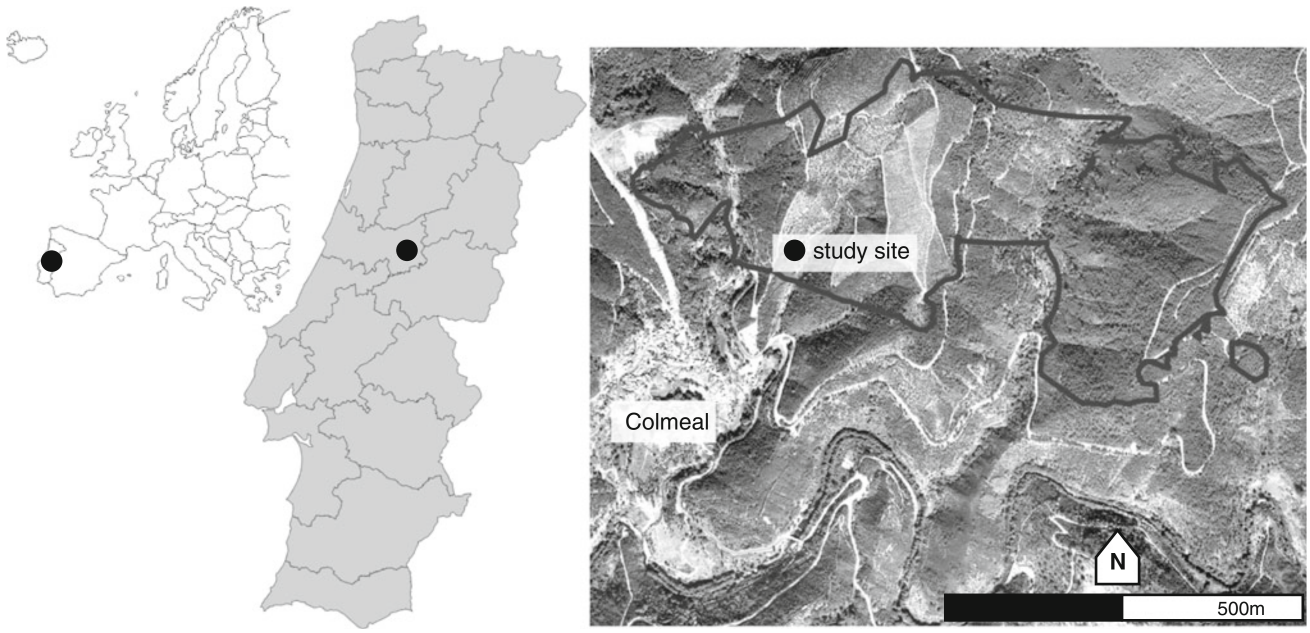

Fig. 1 Location of the Colmeal study area and selected eucalypt plantation instrumented with a slope-scale erosion plot (*black dot*)

patches where canopies were fully consumed (and thus, the ash-covered ground surface gave rise to the dark colour; medium severity). All the stands within the burnt area presented a mixture of light and dark-coloured patches, mostly with a clear predominance of the former. At the five instrumented hill slopes, fire severity was then assessed in more detail along a transect from the bottom to the top of the slope. This involved describing the degree of the tree canopy consumption of 10 randomly selected trees (partial vs. total) as well as the degree of the litter consumption (partial vs. total) and the colour of the ash (black vs. grey) at 5 plots of 50 × 50 cm. In the case of the present study site, the prevalence of partially consumed tree canopies, the total consumption of the litter layer and the black colour of the ashes confirmed the moderate-to-low severity (see e.g. Shakesby and Doerr 2006; Keizer et al. 2008).

Sampling procedures

At the selected slope, overland flow was collected at two occasions, namely on 10 October 2008 after the first significant rainfall event following the wildfire and about 1 year later, on 23 September 2009. This was done by mixing, in roughly equal proportions, the overland flow that had collected in the four 60 l tanks connected to the plot′s four outlets, so to obtain at each sampling occasion a single composite sample of the overland flow from a contributing area of approximately 100 m^2. The sample was then divided over various plastic bottles of 5 l, which, following transport and filtering in the laboratory using glass fiber filters (1.2 μm pore size; APHA-2005), were stored at −18 °C till further processing. Also the filters were stored at −18 °C prior to the analysis of their PAH contents.

PAH analysis

The PAHs included in this study were the sixteen that the United States Environmental Protection Agency (USEPA) identified as priority contaminants. They were the following: naphthalene (NAP), acenaphthylene (ACY), acenaphthene (ACE), fluorene (FLU), phenanthrene (PHE), anthracene (ANT), fluoranthene (FLT), pyrene (PYR), benz(a)anthracene (BaA), chrysene (CHR), benzo(a)pyrene (BaP), benzo(b)fluoranthene (BbF), benzo(k)fluoranthene (BkF), indeno(1,2,3-cd)pyrene (IND), dibenz(a,h)anthracene (DBA) and benzo(g,h,i)perylene (BGP).

The runoff samples were filtered using glass fiber filters a (GF/C, 1.2 μm) to analyse the dissolved and particulate PAH loads separately. The dissolved PAH concentrations of the filtered runoff were analysed using solid-phase micro-extraction (SPME) in a 100 μm poly-dimethysiloxane (PDMS) that was used as the absorbing material. After the extraction, the fiber was desorbed in a gas chromatograph (GC) (Varian CP-3800), with a split/splitless injector. The GC was coupled to a mass spectrometer Ion Trap Saturn 2200 (GC-MS) for the identification of the PAHs. The analytical procedure was validated by doping the sample with standards of the 16 PAHs. The recovery rates of the individual PAHs ranged from 70 % to 117 %. The detection limits (DL) were between 0.21 and 7.31 ng l^{-1}.

The results presented here for each sample were the averages of two independent replicates.

Following drying of the filters at room temperature until constant weight, the particle-bound PAHs were extracted with acetone using microwaves high pressure and their concentrations were determined using the same analytical procedures as describe previously.

Ecotoxicological assays

The ecotoxicological assays were carried out with four aquatic species from different trophic/functional levels that are commonly used for that purpose. They are the bacteria *V. fischeri*, the green algae *P. subcapitata*, the macrophyte *L. minor* and the invertebrate *D. magna*. All four species were exposed to various dilutions of filtered (F) and unfiltered (NF) runoff. All ecotoxicological tests fulfilled the validity requirements established in their respective guidelines (AE 1998; OECD 1998, 2006a, b).

Culture conditions of test species

Unialgal batch cultures of *P. subcapitata* were maintained in 250 ml erlenmeyer with 100 ml of Marine Biological Laboratory medium MBL sterilized Woods Hole Culture (Stein 1973) in an incubator chamber, with controlled temperature (20 ± 2 °C) and photoperiod (16^Lh:8^Dh) and with light provided by cool-white fluorescent lamps. To start new cultures, algae were harvested in the exponential growth phase (5–7 days old) and then inoculated into fresh medium.

The macrophyte *L. minor* was collected in a 250 ml erlenmeyer properly covered with cotton wrapped in gauze to minimize evaporation and accidental contamination and was maintained in laboratory conditions in Steinberg medium (OECD 2006a) during 8 weeks prior to testing. Culture *L. minor* were renewed 2 times per week and maintained in an incubator chamber, with controlled temperature (20 ± 2 °C) and photoperiod (16^Lh:8^Dh) and light intensity of about 6500 lux.

Monoclonal cultures of *D. magna* (clone A, sensu Baird et al. 1989a) were continuously reared under a temperature of 20 ± 2 °C and 16^Lh:8^Dh cycle, in synthetic ASTM hardwater medium (ASTM 1996) supplied with an organic additive extracted from the algae *Ascophyllun nodosum* (Baird et al. 1989b). Cultures were renewed every other day and the organisms fed with *P. subcapitata* (cyclically cultured in Woods Hole MBL medium according with Stein 1973) at a rate of 3.00×10^5 cells ml^{-1}.

Luminescence inhibition test with V. fischeri

The luminescence inhibition test (Microtox test) was performed according to the standard protocol (AE 1998). This involved measuring the decrease in bacterial luminescence following exposure to a series of dilutions of the filtered and unfiltered runoff, involving a single replicate per dilution. For the 81.9 % basic test, the highest concentration tested was 81.9 %. Bacterial luminescence was measured 5 and 15 min after the bacteria had been transferred to the vials samples.

Growth Inhibition test with P. subcapitata

Algal growth assays were conducted according to USEPA (2002b) and OECD (2006b) guidelines. The algae were exposed during 96-h period to several dilutions of filtered and unfiltered runoff water (12.5, 25.0, 50.0, 75.0 and 100.0 %) in MBL medium. To discard any potential effects due to the possible lack of nutrients in highest concentrations tested, assays were performed without nutrients (100 %) and with addition of nutrient according to the formulation of the MBL medium (100 % + N). Assays were performed for three replicates of each treatment plus the control in 100 ml glass vials containing 40 ml of test medium, under constant agitation (≈100 rpm in an orbital shaker), in the same conditions of algal cultures, with an initial cell density of approximately 104 cells ml^{-1}. At the end of the bioassay, the algae cell density (counting of cells on a microscope Olympus CKX41 using a Neubauer chamber) was determined as a biomass parameter (APHA 2005). The endpoints growth rate (GR; day^{-1}) and percentage of growth inhibition (% I) were calculated from the cells density measurements.

Growth inhibition test with L. minor

The growth inhibition test was conducted according to USEPA (2002b) and OECD (2006a) guidelines. The macrophytes were exposed during 7 days to several dilutions of filtered and non-filtered runoff water (12.5, 25.0, 50.0, 75.0 and 100.0 %) in Steinberg medium. In order to exclude any potential effects from nutrient deficiency promoted in the highest concentrations tested, assays were performed without nutrients (100 %) and with addition of nutrient in the formulation of the Steinberg medium (100 % + N).

Assays were performed for three replicates of each treatment plus the control, and colonies consisting of three visible fronds were transferred to 100 ml of testing medium from the inoculum culture and randomly assigned to the test vessels. The number of fronds and colonies was the same in each test vessels (nine fronds per vessel). The test conditions were the same as the culture conditions. The growth rate (GR; day^{-1}) and percentage of growth inhibition (% I) were determined measuring frond number and dry weight at the beginning and after 7 days of exposure.

Reproduction test with D. magna

Chronic reproduction assays with daphnids followed OECD (1998) and USEPA (2002b) guidelines. Runoff samples were tested at several dilutions (12.5, 25.0, 50.0, 75.0 and 100.0 %) performed with ASTM. All the tests started with newborns ageing less than 24 h, born in the bulk cultures between the third and the fifth brood, in order to minimize maternal effects (Barata and Baird 1998). For each treatment and control, ten individual replicates were exposed during 21 days, in 50 ml glass vials, whose test medium was renewed every other day. The test conditions were the same already described for the maintenance of the daphnids, and the animals were fed every 2 days with *P. subcapitata*, at a rate of 3.00×10^5 cells ml^{-1}, supplied with an organic additive. Animals were daily observed for mortality and offspring production being the neonates counted and discharge. The endpoints recorded were survival, growth, fecundity (reproductive output), the age at first reproduction (AFR) and the number of broods. The body size of females was estimated immediately after the release of the first brood, and at the beginning and at the end of the test, by extrapolation from the moult exopodite length (Pereira et al. 2004), allowing the calculation of the somatic growth rate of parent females (SGR, day^{-1}) (Burns 2000). All measurements were made under stereoscope (Olympus SZX9). Fecundity, survival and age at each brood release were integrated for the calculation of the rate of population increase (r, day^{-1}) using the Euler–Lotka equation. The Jackknife technique was used to calculate the standard deviation for r (Meyer et al. 1986).

Nutrient analysis

Nutrient analyses were carried out first and foremost to assess whether nutrient limitations could interfere with the ecotoxicological results for *P. subcapitata* and *L. minor*, that is, to address the possibility that these two species exhibited growth inhibition due to lack of available nitrogen (N) and phosphorous (P) rather than due to ecotoxicological effects. This potential effect of nutrient limitations was especially of interest in the case of the undiluted runoff samples, as dilutions were made with standard solutions with adequate nutrient levels. The concentrations of un-ionized ammonia (NH_3–N, i.e. the most toxic form of nitrogen for aquatic organisms (Koukal et al. 2004); Nessler method), nitrate (NO_3^-; cadmium reduction method), nitrite (NO_2^-; calorimetric method) and soluble ortho-phosphate (PO_4^{3-}; ascorbic acid method) were measured.

The nutrient concentrations of the unfiltered and filtered samples of both sampling periods are given in Table 1. All NO_3^- and NO_2^- values were below their detection limits (0.10 and 0.01 mg l^{-1}, respectively), whilst the NH_3 values only exceeded detectable amounts immediately after the fire. The PO_4^{3-} values did not only change by filtering but also with time since fire. Both changes were of the same sign as well as order of magnitude, corresponding to reductions by 60–65 %. In the case of the NH_3 values for October 2008, the reduction by filtering was less pronounced, amounting to about 15 %.

Table 1 Nutrients concentrations of filtered and unfiltered runoff collected in October 2008 and September 2009 from a recently burnt eucalypt plantation

Nutrients (mg l^{-1})	October 2008		September 2009	
	Unfiltered	Filtered	Unfiltered	Filtered
PO_4^{3-}	1.91	0.68	0.64	0.26
NO_3^-–N	<0.10	<0.10	<0.10	<0.10
NO_2^-–N	<0.01	<0.01	<0.01	<0.01
NH_3–N	1.20	1.00	<0.10	<0.10

The nutrient concentrations in both filtered and unfiltered runoff were considerably below the levels of available N and P that provide optimal growth conditions for either *P. subcapitata* (OECD (2006b): 3.93 mg N l^{-1}; 0.29 mg P l^{-1}) or *L. minor* (OECD (2006a): 83 mg N l^{-1}; 23 mg P l^{-1}). Therefore, an additional treatment was included in the experiments with both species, as also done by for example, Moreira-Santos et al. (2004). It involved the addition of nutrients to the undiluted runoff samples. It should be noted, though, that *L. minor* can grow under a markedly wide range of nitrogen and phosphorous concentrations (Landolt and Kandeler 1987). According to these authors, the minimal nutrient levels for *L. minor* to achieve half of its maximum growth rate are 0.07 mg N l^{-1} and 0.0034 mg P l^{-1}, whereas the maximum levels that *L. minor* can tolerate are 375 mg N l^{-1} and 154 mg P l^{-1}.

Data analysis

The effect concentration that produced a reduction in the measured endpoints of the test organisms with 50 %—the so-called EC_{50}—was estimated using a special-purpose software in the case of *V. fischeri* (Microtox Omni™, version 4.3.0.1; AE 1998), and using Statistica 8.0 in the case of the other three test species. In Statistica, EC_{50} and its 95 % confidence limits were estimated by non-linear regression using the logistic equation: $v_{2i} = c/(1 + (v_{1i}/EC_{50})^{**}b$, where: v_{2i} is growth rate in the case of *P. subcapitata* and *L. minor*, and somatic growth rate and rate of population increase in the case of *D. magna* at concentration *i* of the runoff sample; *c* is the maximum value within the control treatment; v_{1i} is the concentration *i* of the runoff sample; *b* is the slope of the line fitted through the data using the least squares method.

One-Way ANOVA was applied to test whether there existed significant differences, at $\alpha = 0.05$, in (somatic)

Table 2 PAHs concentrations in ng l^{-1} [mean ± standard deviation ($n = 2$)] of the dissolved and particulate phases of runoff samples collected in October 2008 and September 2009 at a recently burnt eucalypt stand

PAHs	Rings	October 2008		September 2009	
		Dissolved phase (ng l^{-1})	Particulate phase (ng l^{-1})	Dissolved phase (ng l^{-1})	Particulate phase (ng l^{-1})
NAP	2	14.8 ± 0.3	284 ± 105	24.5 ± 1.6	137 ± 6
ACY	3	<DL	343 ± 116	<DL	<DL
ACE	3	<DL	<DL	<DL	<DL
FLU	3	5.32 ± 0.35	41.5 ± 7.81	<DL	30.9 ± 0.1
PHE	3	<DL	42.4 ± 11.0	1.68 ± 0.18	37.7 ± 0.3
ANT	3	<DL	<DL	<DL	<DL
FLT	4	<DL	10.8 ± 0.6	1.22 ± 0.04	8.00 ± 0.61
PYR	4	1.86 ± 0.45	11.6 ± 2.8	1.21 ± 0.02	3.35 ± 0.16
CHR	4	14.6 ± 2.8	81.6 ± 10.5	14.3 ± 1.0	74.6 ± 0.8
BaA	4	53.4 ± 20.1	290 ± 17	<DL	<DL
BbF	5	<DL	<DL	0.98 ± 0.08	1.94 ± 0.18
BkF	5	<DL	<DL	1.99 ± 0.16	4.78 ± 0.43
BaP	5	<DL	<DL	<DL	<DL
DBA	5	<DL	<DL	<DL	<DL
BGP	6	<DL	<DL	<DL	3.38 ± 0.11
IND	6	<DL	<DL	<DL	4.42 ± 0.29
ΣPAHs		90.0	1,104	45.9	306

<DL stands for below detection limit

growth rate (*P. subcapitata*, *L. minor*, *D. magna*) and the rate of population increase (*D. magna*) amongst the control treatment and the different dilutions of the runoff samples (Zar 1996). This was done using Sigmaplot 11.0 and included verification of the underlying assumptions of normality and homoscedasticity with the Shapiro–Wilk test and the Levene Median test, respectively. In the case of significant differences, Dunnett's test was employed to test which treatment was significantly different to the control treatment ($\alpha = 0.05$), and this information was then used to determine the lowest concentration of the runoff sample revealing an effect on species performance (LOEC).

The null hypothesis that nutrient limitations did not significantly ($\alpha = 0.05$) affect the growth rates of *P. subcapitata* or *L. minor* in the undiluted runoff samples (see 2.4.6.) was tested using Student's *t* test, as it concerned a contrast between two treatments ('100 %' vs. '100 % + N'). This was done with Sigmaplot 11.0 and, as before, included the verification of the underlying assumptions of normality and homoscedasticity.

Results and discussion

PAHs concentrations

The total loads of the 16 selected PAHs (ΣPAHs) dropped markedly between October 2008 and September 2009 (Table 2). Presumably, this reduction was first and foremost due to a reduced availability of ash deposits, as was also indicated by Olivella et al. (2006). In the case of the dissolved phase, the decrease was about half, whereas in the case of the particulate phase, it was almost three quarters. As a result, the predominance of the particulate over the dissolved fraction (reflecting the high affinity of PAHs for suspended particles) was less pronounced 1 year than immediately after the wildfire (i.e. with a factor 6 vs. 12). Such a decrease in the particulate/dissolved ratio with time since fire was also suggested by the results of Vila-Escalé et al. (2007), dropping from 95 to less than 2 between 12 and 445 days after fire. Unlike in the present case, however, it reflected first and foremost a reduction in the total concentrations of the particulate phase (from 369 to 7.1 ng l^{-1}), since the total dissolved concentrations were basically the same at these two sampling dates (3.9 vs. 4.3 ng l^{-1}). The discrepancy with the present study could be due to the different sampling objects, that is, the water samples of Vila-Escalé et al. (2007) were collected in a depositional pond at the downstream limit of a burnt catchment (in north-east Spain). This difference in sampling universe could also contribute to the markedly lower total concentrations in Vila-Escalé et al. (2007) than in this study, for both the dissolved and particulate fraction. A further difference in sampling object could also help explain why the October 2008 values differed even more from the total values obtained by Olivella et al. (2006) after

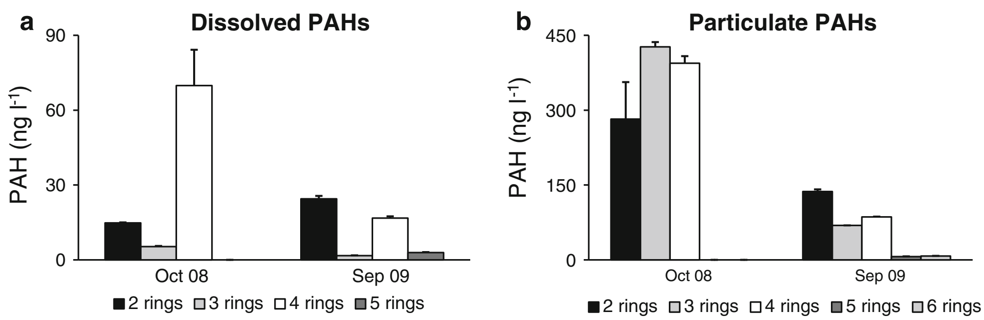

Fig. 2 The composition pattern of PAHs by ring size in dissolved phase (**a**) and particulate phase (**b**) in runoff water samples collected in October 2008 and September 2009 from a recently burnt eucalypt plantation. *Error bars* represent standard error ($n = 2$)

the first, heavy post-fire rainfalls in two other burnt watersheds in north-east Spain (12 PAHs, dissolved + particulate phase: 0.3–37 ng l^{-1}). Namely, Olivella et al. (2006) not only studied riverine water samples but these samples also revealed a considerable dilution effect due to the rainfall.

The composition pattern of PAHs by ring size revealed a more heterogeneous make-up for the dissolved than particulate phase, especially immediately after the wildfire (Fig. 2). The 4-ring PAHs (MMW—Medium Molecular Weight) clearly dominated the dissolved phase in October 2008 (approx. 80 %), whilst the 3-ring PAHs were underrepresented in the dissolved phase at both sampling occasions (4–6 %). High Molecular Weight PAHs (HMW: 5 or 6 rings) were not detected in the October 2008 sample but amounted to approximately 5 % in the September 2009 sample, in both the dissolved and particulate phase. Also the 2-ring PAHs revealed consistently higher contributions 1 year than immediately after the wildfire, even becoming the predominant fraction of the dissolved as well as particulate phase with roughly 50 %. Possibly, the predominance of naphthalene in the PAHs profiles 1 year after the fire derived from intrinsic factors, like biogenic synthesis (Meire et al. 2008), in combination with its elevated solubility. This could explain the marked increase in naphthalene's concentrations in the dissolved phase, whilst the concurrent decrease in the particulate phase would reflect the reduced availability of ashes. Also, 'fresh' inputs of naphthalene by rainfall could play a role (Brun et al. 2004). The present results were quite different from those of Vila-Escalé et al. (2007), both for the dissolved and particulate phase. In the case of the dissolved phase, the 3-ring PAHs were the dominant fraction in the samples of Vila-Escalé et al. (2007) during the first 122 days after the fire, contrary to what was the case in this study. Also, the HMW PAHs were found by Vila-Escalé et al. (2007) from the first sampling date onwards. The same was true for the particulate phase. A further difference regarding the particulate phase was that the 4-ring PAHs clearly dominated over the entire period studied by Vila-Escalé et al. (2007). In the study of Olivella et al. (2006) in which the dissolved and particulate phase were analysed together, the 3-ring PAHs tended to predominate after the first post-fire rainfall. This would agree well with the results of Vila-Escalé et al. (2007) for the dissolved phase, assuming that the dilution effect caused by rainfall observed by Olivella et al. (2006) affected especially the particulate phase.

In terms of the individual PAHs, four compounds dominated the different samples (Table 2). Two of them—NAP (16–53 %) and CHR (7–31 %)—were principal components at both sampling occasions, whereas the two other—BaA (26–59 %) and ACY (31 %)—were dominant immediately after the fire but below the detection limit 1 year later. The dissolved phase revealed a striking shift in dominance with time since fire, from BaA (59 %) to NAP (53 %). NAP (45 %) also clearly dominated the particulate phase 1 year after fire. Immediately after the fire, however, NAP (26 %) contributed to the particulate PAHs basically to the same extend as ACY (31 %) and BaA (26 %). Worth stressing is perhaps that BaA is one of the most potent carcinogenic PAHs. As to be expected from the above-mentioned differences in ring-based composition, the results presented here were markedly different from those of Vila-Escalé et al. (2007). A point of agreement, however, was that NAP was also one of the principal compounds of the dissolved phase immediately after the fire studied by Vila-Escalé et al. (2007). In the case of Olivella et al. (2006), PHE tended to be the principal compound after the first post-fire rainfall. Nonetheless, even their three highest concentrations (1–6 ng l^{-1}) were well below the present values (dissolved + particulate: 39–42 ng l^{-1}).

Toxicity tests

Luminescence inhibition test with V. fischeri

According to the Microtox basic test, the runoff collected immediately after the fire as well as 1 year later was highly toxic to *V. fischeri* (Fig. 3; Table 3). In the case of the October 2008 sample, a toxic effect was only observed in the unfiltered runoff. By contrast, filtering did not avoid such a detrimental impact in the case of the September 2009 sample. Also the role of exposure time on the observed toxicity contrasted for the two sampling periods.

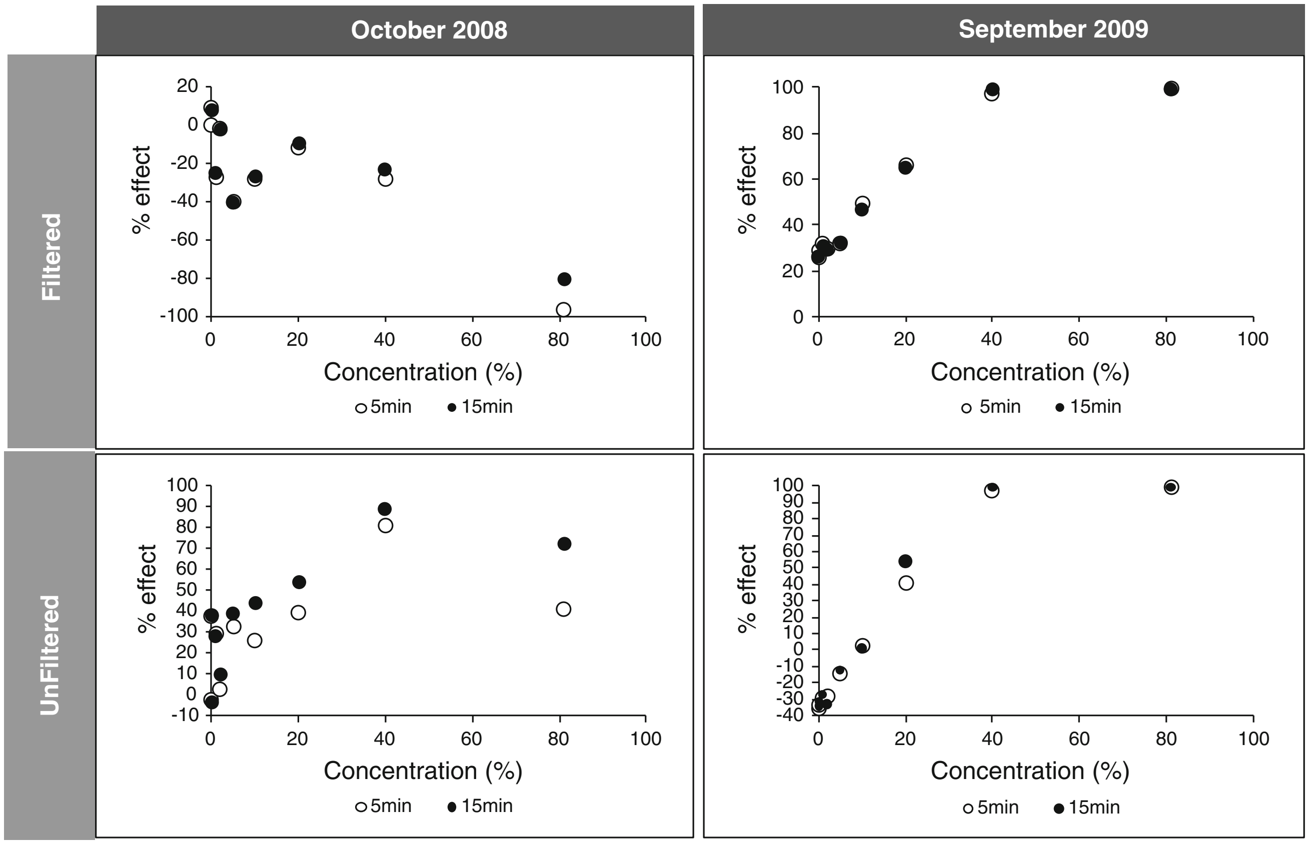

Fig. 3 Microtox luminescence inhibition of *V. fischeri* exposed to different concentrations of filtered and unfiltered runoff collected at a recently burnt eucalypt stand in October 2008 and September 2009

Toxicity increased with increasing exposure time from 5 to 15 min for the (unfiltered) runoff collected immediately after fire, whilst the opposite was true for the (filtered) runoff collected 1 year later. The toxicity of PAHs for *V. fischeri* was also observed by Bihari et al. (2007), using PAH-contaminated seawater.

Growth inhibition test with P. subcapitata

The growth rate of *P. subcapitata* was not only significantly but also strongly reduced (80–100 %) by the undiluted runoff from the burnt eucalypt plantation (Fig. 4; Table 4). This marked effect was observed independently of the filtering of the samples as well as of the time since fire. Even so, the sample of September 2009 appeared to provoke a stronger response than that of October 2008. This was suggested by the stronger growth reduction in the case of the undiluted samples [100 vs. 80 %, the associated EC_{50} values being 60 vs. 90 (Table 5)] but especially of the 25 %-diluted samples (90–100 vs. 15–20 %). Possibly, this tendency for an enhanced growth inhibition with time since fire was due to the above-mentioned changes in PAH composition, in particular the increased fraction of naphthalene, Namely, naphthalene is relatively water-soluble and, thus, easily available to species such as *P. subcapitata* (Kayal and Connel 1995; Baumard et al. 1999a). Also the remaining dilutions of both sampling periods decreased the growth rate of *P. subcapitata*. This impact, whilst consistent, was minor (<20 %) and only statistically significant in the case of the filtered runoff collected in October 2008, giving a comparatively low LOEC value of ≤12.5 %.

The addition of nutrients to the undiluted samples (‘100 % + N’ treatment) attenuated significantly the growth rate inhibition of *P. subcapitata* (Fig. 4; Student’s *t* test: $p = <0.001–0.03$). This was true for the runoff of both sampling dates and for the filtered as well as unfiltered samples. In terms of the EC_{50} parameter, nutrient addition produced about 10 % higher values in the case of the October 2008 as well as the September 2009 samples (Table 5). The positive effect of additional nutrients was in line with the measured nutrient concentrations in the runoff samples (see Table 1), being below the optimal growth levels of *P. subcapitata* (OECD 2006b). Thus, adequate nutrient levels enhanced the species’ tolerance to stressors, a phenomenon that was also observed by Moreira-Santos et al. (2004). Nonetheless, the compensatory role of the adequate nutrient levels was only effective to a certain degree, since the ‘100 + N’ treatments resulted in

Table 3 EC_{50} (%) values of the bacteria *V. fischeri* exposed to runoff samples collected at a recently burnt eucalypt stand in October 2008 and September 2009

Sample	Matrix	EC_{50} (%)	
		5 min	15 min
Oct 08	Unfiltered	10 (1–83)	7 (2–25)
	Filtered	NT	NT
Sep 09	Unfiltered	ND	ND
	Filtered	3 (1–9)	8 (2–24)

The values between brackets represent the 95 % confidence limits, and 'NT' and 'ND' stands for-no toxicity and not determinate, respectively

significantly lower growth rates than the control treatments in all four instances ($p < 0.05$).

Growth inhibition test with L. minor

L. minor exhibited a very similar response to the sample collected 1 year after the wildfire as *P. subcapitata* did (Fig. 4; Tables 4 and 5). Namely: (1) the undiluted and 25 %-diluted samples reduced the growth rate of *L. minor* to practically zero, whilst the greater dilutions had clearly less impact; (2) filtering had no marked effect on this reduction in growth rate; (3) addition of nutrients significantly attenuated these toxic effects but only partially, so that the response continued significantly different from that to the control sample. Notwithstanding these similarities, *L. minor* showed greater sensitivity to the September 2009 sample than *P. subcapitata*, as evidenced by the lower LOEC values (≤25 vs. 75 %) as well as the lower EC_{50} values (38–53 vs. 60–74 %).

By contrast, *L. minor* was less sensitive to the sample collected immediately after the wildfire than *P. subcapitata* in that the undiluted samples reduced the growth rate of *L. minor* to a markedly lesser—albeit still statistically significant—extent (approximately 20 vs. 80 % for *P. subcapitata*). By implication, *L. minor* also revealed a stronger contrast in its response to the two sampling periods than *P. subcapitata*, as also evidenced by the greater differences in EC_{50} values between the two sampling dates for both the filtered and unfiltered samples. In line with the explanation suggested in the previous section, *L. minor* would seem even more sensitive than *P. subcapitata* to the composition rather than to the total load of PAHs, in particular to the fraction of naphthalene presumably due to its comparatively high availability for uptake (Kayal and Connel 1995;

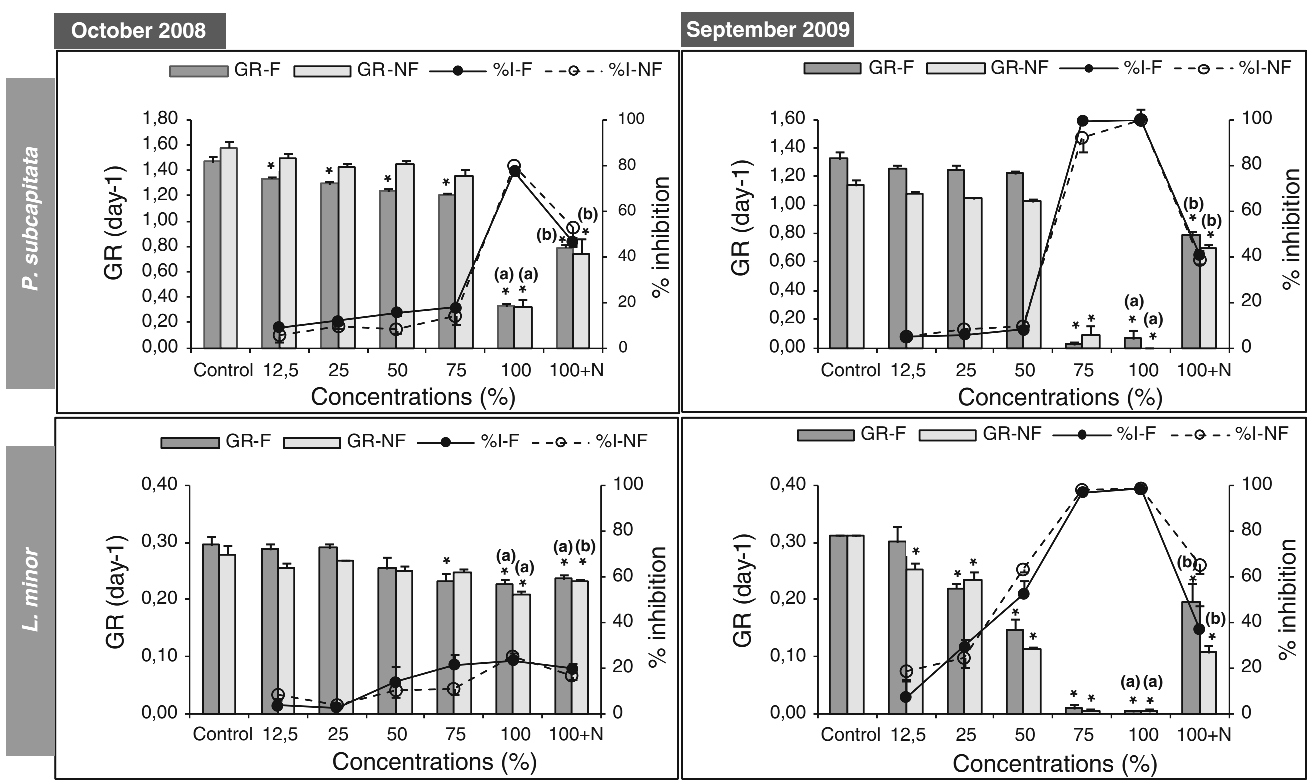

Fig. 4 Growth rates (GR) and % of inhibition (%I) for *P. subcapitata* and *L. minor* exposed to different dilutions of filtered (F) and unfiltered (NF) runoff collected at a recently burnt eucalypt stand in October 2008 and September 2009. *Errors bars* represent standard error, *asterisks* indicate significantly differences from the control treatments, and the *letters a* and *b* indicates significant differences between the undiluted samples with additional nutrients ('100 + N') and without ('100')

Table 4 One-way ANOVA results and LOEC values (%; $p < 0.05$) (%) for the growth rates (GR) of *P. subcapitata* and *L. minor*, and for the life-history endpoints of somatic growth rate (SGR) and intrinsic population increase (r) in *D. Magna*, exposed to filtered (F) and unfiltered (NF) runoff collected at a recently burnt eucalypt plantation in October 2008 (Oct 08) and September 2009 (Sep 09)

Species	Endpoint	Sample		*df*	MS_{res}	F_{ratio}	*H*	p_{value}	LOEC
P. subcapitata	GR	Oct 08	NF	6	$1.09e^{-2}$	62.283	–	<0.001	100
			F	6	$1.95e^{-3}$	243.393	–	<0.001	≤12.5
		Sep 09	NF	6	$3.17e^{-3}$	224.050	–	<0.001	75
			F	6	$4.34e^{-3}$	229.177	–	<0.001	75
L. minor	GR	Oct 08	NF	6	$2.03e^{-4}$	7.848	–	<0.001	100
			F	6	$4.98e^{-4}$	5.460	–	0.004	75
		Sep 09	NF	6	$2.31e^{-4}$	197.300	–	<0.001	≤12.5
			F	6	$9.83e^{-4}$	48.029	–	<0.001	25
D. magna	SGR	Oct 08		6	–	–	17.349	0.008	ND
	r			6	–	–	31.086	<0.001	ND
	SGR	Sep 09		6	$6.65e^{-5}$	2.324	–	0.045	ND
	r			6	–	–	16.929	0.01	ND

ND stands for not determinate

Table 5 EC_{50} values (% dilution) and respective 95 %-confidence limits at for the growth rates (GR) of *P. subcapitata* and *L. minor*, and the life-history endpoints of somatic growth rate (SGR) and intrinsic rate of population increase (r) in *D. magna*

Species	Endpoint	Sample		EC_{50} (%)	
				Unfiltered	Filtered
P. subcapitata	GR	Oct 08	100	90.3 (87.1–93.5)	90.8 (87.1–94.3)
			100 + N	99.9 (95.4–104.5)	>100
		Sep 09	100	62.2 (58.2–66.3)	59.8 (53.3–66.3)
			100 + N	74.0 (40.9–107.1)	69.7 (41.9–97.5)
L. minor	GR	Oct 08	100	>100	>100
			100 + N	>100	>100
		Sep 09	100	41.8 (35.8–47.8)	40.8 (31.8–49.7)
			100 + N	38.3 (24.8–51.8)	52.8 (11.7–93.8)
D. magna	SGR	Oct 08		NT	NT
	r			NT	NT
	SGR	Sep 09		NT	NT
	r			NT	NT

The '100 + N' treatment involved the addition of nutrients, and 'NT' stands for not toxic

Baumard et al. 1999a). This could also explain why in the case of *L. minor* the attenuation effect of adding nutrients was only significant in the case of one of the two October-2008 samples as opposed to both September 2009 samples (Fig. 4; Student's *t* test: $p = 0.001$–0.462). On the other hand, the lack of a positive effect of nutrient addition could indicate that the growth *L. minor* was not as seriously nutrient limited as that of *P. subcapitata*, possibly because the species' marked tolerance to sub-optimal nutrient concentrations as those measured in the undiluted runoff samples (Landolt and Kandeler 1987; OECD 2006a).

Reproduction test with D. magna

Unlike was the case for the other three test species of lower trophic levels, *D. magna* did not reveal significant inhibitory effects to either the runoff collected immediately after the wildfire or that collected 1 year later (Fig. 5; Tables 4 and 5). As reviewed in ECHA (2009), it is well documented that daphnids can be less sensitive to contaminated water than species of lower trophic levels. Possibly, the daphnids in this study were able to modify their energy allocation patterns to permit their maintenance and survival under more adverse conditions, as also suggested by

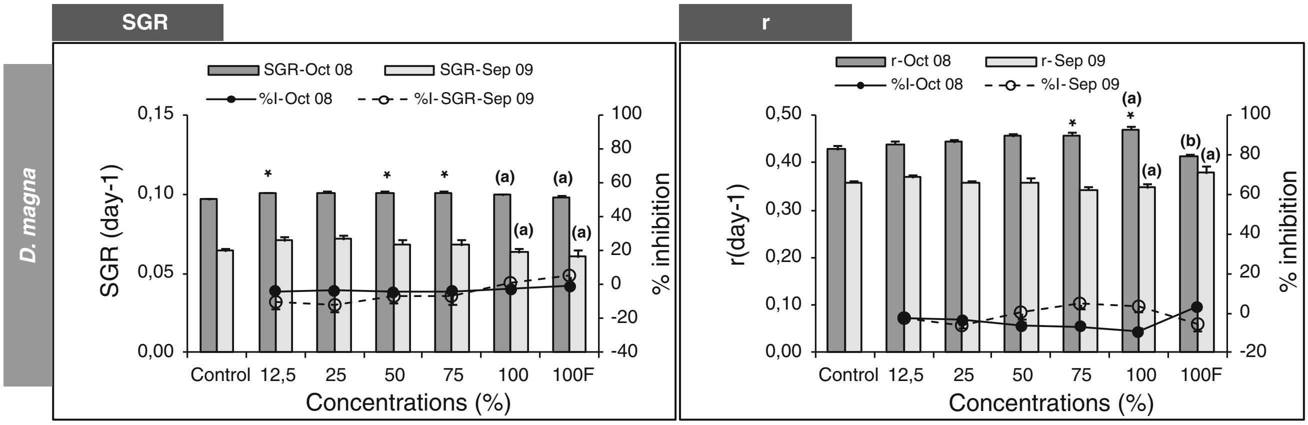

Fig. 5 Somatic growth rate (*SGR*, day^{-1}) and intrinsic rate of population increase (*r*, day^{-1}) in *D. magna* exposed to different concentrations of runoff water samples collected at a recently burnt eucalypt stand in October 2008 and September 2009, where '100F' stands for filtered undiluted sample. *Errors bars* represent standard error, *asterisks* indicate significantly differenced from the control conditions ($\alpha \leq 0.05$), and the *letters a* and *b* indicate significant differences between the filtered and unfiltered, undiluted samples '100F' and '100', respectively; $\alpha \leq 0.05$)

Polishchuk and Vijverberg (2005) and Pieters and Liess (2006). Furthermore, the daphnids might have improved their fitness by extending their diet to include dissolved or suspended nutrients (Roche 1998; Nandini et al. 2005), particulate organic matter (Antunes et al. 2007a) and/or bacteria (Lampert 1987), especially the present study concern natural runoff samples (Peterson et al. 1978; Jürgens 1994; Langenheder and Jürgens 2001; Degans et al. 2002; Xuwang et al. 2011). Bioassays carried out with natural samples have frequently revealed positive rather than the expected negative responses on cladoceran life-history traits (e.g. Antunes et al. 2007a, 2007b; Abrantes et al. 2008, 2009; Marques et al. 2011). The October 2008 sample did, in fact, show some significant increases in the rates of somatic as well as population growth. They were more likely related to consumption of algae and/or particulate organic matter than of nutrient, as the nutrient concentrations were rather low (see Table 1). The role of the particulate phase was also suggested by the fact that filtering significantly decreased population increase to levels similar to control conditions as well as by the fact that the October 2007 sample had a much higher particulate PAH load than the September 2009 sample. Besides to such compensatory phenomena, the lack of growth-reducing effects could simply be due to the relatively low PAH concentrations observed in this study. Namely, none of the 16 PAHs attained concentrations in either the filtered or unfiltered runoff samples exceeded 350 ng l^{-1}, whilst Lampi et al. (2006) and Alvarez et al. (2008) reported 48-h median lethal concentrations (48-h LC50) of individual PAHs for *D. magna* that ranged from 1,040 ng l^{-1} in the case of BGP and 699,000 ng l^{-1} in the case of PHE.

Overall discussion

The results of this study amply justify concerns about the off-site environmental impacts of wildfires, with three of the four test species revealing significant inhibitory effects when exposed to overland flow from a recently burnt area. Especially, in the context of the ongoing implementation of the EU Water Framework Directive (WFD), recently burnt areas should thus not be ignored as potential point sources of pollution for downstream surface water bodies. Whilst the present findings agreed with the decrease in PAH loads with time since fire reported by prior studies (Olivella et al. 2006; Vila-Escalé et al. 2007), they also evidenced that environmental risks are not necessarily limited to the immediate post-fire situation and, thus, would need to be monitored for more than at least 1 year.

For an adequate assessment and monitoring of fire-induced pollution risks, however, much research is needed to further the knowledge and understanding of various key aspects, related with the characteristics of the fire, the sediment transport processes through the hydrographic network, the biogeochemical cycle of the pollutants as well as their ecotoxicological effects. Worth mentioning in this respect is perhaps that some of the important limitations of the present work are now being addressed by a follow-up study, extending the sampling universe in terms of study sites, land-cover types, sampling objects (soil, ashes and sediments), sampling period and frequency as well as broadening the ecotoxicological testing to include, for example, chronic bioassays with *D. magna.* Such chronic bioassays are expected to reveal that the fire-induced effects on organism of higher trophic levels (including humans) are first and foremost indirect, via the propagation of toxic effects through the food web by bottom-up mechanisms (Abrantes et al. 2008).

A major challenge for the follow-up work on the eco-toxicological effects of ash-loaded runoff will be to address the complexity of influencing factors, especially also for test species of the higher trophic levels due to bioaccumulation processes (e.g. Bícego et al. 2006). This was illustrated well by the present study in that it proved difficult to pinpoint the observed inhibitory effects to specific aspects of the PAH loads of the tested runoff samples. A first reason was that the samples, as seems to be typical in burnt areas (Olivella et al. 2006; Vila-Escalé et al. 2007), consisted of mixtures of multiple (predominant) compounds, so that the role of possible synergistic, antagonistic or additive effects between the various PAHs could not be precluded (Pardos et al. 1998). Second, the sample with the clearly highest PAH load—that is, collected in October 2008 after the first post-fire rains—appeared to inhibit the three species of the lower trophic levels to a lesser extent than the sample collected 1 year later. Third, filtering supposedly removed about 90 % of the overall PAH load but this did not substantially attenuate the inhibitory effect of the runoff, except in one of six cases (that of the October 2008 sample on *V. fischeri*). A possible explanation for the limited role of the particulate phase was that the PAHs were strongly absorbed to the particles and, thus, poorly ‘available’ to the test species, especially since the particulate phase consisted to a large extent of charred organic matter (see Malvar et al. 2011). In a real-world situation, however, this particulate phase could be a chronic source of PAH contamination in depositional environments, also because PAHs are relatively stable compounds, especially also under anaerobic conditions (e.g. Mihelcic and Luthy 1988; Kim et al. 1999).

In line with the above-mentioned, any explanation for the most unexpected finding of this study—the greater effects of the runoff collected 1 year than immediately after the fire—can at best be tentative. An obvious factor that could be involved was naphthalene or, to be more specific, either the relative amounts of naphthalene in the total loads of dissolved and particulate PAHs (45–50 vs. <25 %) or the absolute dissolved naphthalene concentration (25 vs. 15 ng l^{-1}). The importance of the dissolved naphthalene concentration would agree especially well with the fact that LMW PAHs (2–3 rings) and especially naphthalene are more water-soluble than MMW PAHs (4 rings), and, thus, more easily ‘available’ to species like *P. subcapitata* and *L. minor* (Kayal and Connel 1995; Baumard et al. 1999a, 1999b). Furthermore, LMW PAHs tend to produce a stronger acute toxicity than MMW or HMW PAHs (5–6 rings) (Law et al. 1997; Boonchan et al. 2000). On the other hand, the concentrations reported here were well below the concentrations, at which individual LMW PAHs were found to inhibit growth and cell division in aquatic bacteria and algae (5–100 µg l^{-1}), let alone to impair their cell division and ultimately cause their death (0.2–10 mg l^{-1}) (Eisler 2000). This discrepancy highlights the possible existence of combined effects in the present samples with their highly variable mixtures of distinct PAHs. Such combined effects are notoriously difficult to predict on the basis of individual toxicities of indentified compounds (Pardos et al. 1998; Brack 2003; Wenzl et al. 2006).

Conclusions

The principal conclusions of this study into the ecotoxicological effects of overland flow collected at a recently burnt eucalypt stand in north-central Portugal immediately after a low-medium severity wildfire and 1 year later were the following:

1. The total amounts of 16 priority polycyclic aromatic hydrocarbons (PAHs) in the collected runoff samples were high compared with the values of prior studies in burnt areas, probably due to differences in sampling objects (hill slope runoff in this study vs. riverine and depositional ponds);
2. The dissolved as well as particulate PAH loads were markedly higher following the first rainfall event after the wildfire than 1 year later and also had noticeably different compositions in terms of ring-based as well as individual PAHs;
3. The runoff produced statistically significant and, in general, conspicuous inhibitory effects on three of the four test species, that is, on the bacteria *V. fischeri*, the green algae *P. subcapitata* and the macrophyte *L. minor* but not on the invertebrate *D. magna* representing the highest trophic level;
4. Surprisingly, the runoff collected immediately after the wildfire was less toxic to *V. fischeri*, *P. subcapitata* and *L. minor* than the runoff collected 1 year later, suggesting that total loads of PAHs can be less important than their composition, and clearly demonstrating that the off-site effects of wildfires are not necessarily limited to the period immediately after the fire.
5. Further research is urgently needed to provide a sound scientific basis for assessing, monitoring and predicting the risks of surface water pollution by recently burnt areas, including with respect to the ‘window-of-ecotoxicological effects’ by runoff from recently burnt areas, the role therein of individual and mixtures of PAHs, and the propagation of toxic effects from the lower to the upper trophic levels.

Acknowledgements This study was carried out with logistic support of the EROSFIRE-II project, funded by the Portuguese Foundation for Science and Technology (FCT) with co-funding by FEDER through the POCI2010 Programme (PTDC/AGR-CFL/70968/2006). IC and NA gratefully acknowledge their research grants by the FCT with references SFRH/BD/ 62327/2009and SFRH/BPD/35665/2007, respectively. Also, the anonymous reviewer is gratefully acknowledged for his/her help in improving this manuscript.

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