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RESEARCH ARTICLE

Genotoxicity in the rivers from the Brantas catchment (East Java, Indonesia): occurrence in sediments and effects in *Oreochromis niloticus* (Linnæus 1758)

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Abstract

This paper reports the first data from an integrated study investigating genotoxicity in the Brantas River, Java, Indonesia. Results showed that organic sediment extracts from the sites in the Brantas Delta retained genotoxic compounds identified using the SOS Chromotest and that the Aloo River and, to a lesser extent, the Surabaya River were the most contaminated studied sites. This genotoxicity was attributable to compounds that did not require any bioactivation under the test conditions. Occurrence of genotoxic effects was further investigated in erythrocytes from Nile tilapia, *Oreochromis niloticus*. High numbers of micronuclei were counted, especially in fish sampled in the rivers of the Brantas Delta. Moreover, cytoplasmic alterations which could be indicative of the presence of lipofuscin were found in the cytoplasm of the fish blood cells, especially in fish from the Aloo, Surabaya and Kalimas rivers. Altogether, our data showed that genotoxicity is occurring in fish living in rivers of the delta of the Brantas River and suggest that sediments from these sites may constitute a major source of pollution and hazard for species living or feeding in the area.

Keywords Brantas River · Genotoxicity · SOS Chromotest · Micronuclei · Fish · Nile tilapia

Introduction

A large number of chemicals contaminating the environment have carcinogenic or mutagenic effects. Many of them, especially PAHs, can partition into the sediment in the aquatic environment (Narbonne et al. 1999; Metcalfe et al. 1990; Chen and White 2004). Aquatic organisms are then exposed to this contamination and subject to DNA or cellular damages which can affect the population and the ecosystem by passing through the trophic chain (Izquierdo et al. 2003; Diekmann et al. 2004). Accordingly, genotoxic metabolites have been found in various aquatic and marine organisms (Wessel et al. 2010; Bolognesi et al. 2006; Baršienė et al. 2006; Floehr et al. 2015).

Various tests can be performed to evaluate the genotoxicity. Among others are the SOS Chromotest and the micronucleus (MN) test. The SOS Chromotest has been tested with a large number (> 700) of known genotoxic compounds and found very sensitive (Quillardet and Hofnung 1993). Bacterial assays, including the SOS Chromotest, have also been widely used to assess the genotoxicity of suspended or bottom sediments (reviewed in Chen and White 2004) and used in monitoring studies (Chapman 1990; White et al. 1998; Cachot et al. 2006; Floehr et al. 2015). The MN assay is described by the OECD (test nos. 474 and 487) and is recommended by the International Conference on Harmonization (ICH 1997), the Food and Drug Administration and other regulatory

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agencies to investigate the genotoxic and chemopreventive potential of new agents (Cimino 2006). A correlation between an increase in micronuclei frequency and the development of carcinogenesis has been documented thus demonstrating the good predictive value of the MN test for carcinogenicity (Jenssen and Ramel 1980; Olaharski et al. 2005). The MN assay has been applied in different cell types of fishes (Al-Sabti and Metcalfe 1995; Arkhipchuk and Garanko 2005; Seriani et al. 2011) and deployed in field monitoring studies using several fish species including the Nile tilapia (Çavaş and Ergene-Gözükara 2005; Ozkan et al. 2011; Da Rocha et al. 2011).

This study aimed at investigating the presence and effects of genotoxic compounds in the Brantas River, the longest river in Indonesia which divides into several other rivers in its large delta. The Brantas catchment covers about 12,000 km^2 and is hosted by 14 million inhabitants (Aldrian and Djamil 2008). The study area covers four rivers in the Brantas Delta of East Java: the Kalimas and the Surabaya rivers located in the industrial and urban metropolitan city of Surabaya and the Aloo and the Porong rivers which are impacted by the release of volcano mud since 2006. The history of pollution on this location has been reported earlier (Davies et al. 2008 and 2011). As an essential step prior to measuring organisms' responses, we sought to better characterize the genotoxicity of the sediment compartment. It is the main reservoir of particle-associated contaminants in aquatic ecosystems and one of the main sources of contamination for biota. Effects were investigated in the commonly consumed fish *Oreochromis niloticus* (Perciformes, Cichlidae) known by the local population in Indonesia as "ikan Nila" or commercially known as Nile tilapia. This species has already been used in genotoxicity and mutagenicity studies because of its sensitiveness to a variety of contaminants (Palhares and Grisolia 2002). Effects were investigated in blood cells as blood has been proven to be a sensitive compartment for genotoxicity measurement in fish (Rocha et al. 2009; Boettcher et al. 2010). Our previous study showed that anthropogenic and volcano mud pollutions alter innate immune system and weaken fish health as shown by reduced phagocytic activity in fishes (Risjani et al. 2014).

Material and methods

Study area

This study was carried out in the Brantas River, East Java, Indonesia. The Brantas is the longest river in East Java, flowing 320 km from the spring river at Arjuno Mount to river mouths at the Strait of Madura. The Brantas Delta is composed of several rivers and, among them four rivers were investigated, the Surabaya River, the Kalimas River, the Porong River and the Aloo River. Upstream sites were also investigated, including the source of the Brantas (Sumber Brantas) and the Karangkates reservoir (Fig. 1).

Sediment, water and fish collection

Sediments were sampled from December 2011 to January 2012 in Kalimas (7° 17′ 19.7″ S; 112° 44′ 38.8″ E), Surabaya (7° 17′ 59″ S; 112° 44′ 12.7″ E), Porong (7° 32′ 43.5″ S; 112° 43′ 36.4″ E), Aloo (7° 30′ 58.9″ S; 112° 44′ 07.5″ E), Sumber Brantas (7° 44′ 25.5″ S; 112° 32′ 02.2″ E) and Karangkates (8° 10′ 08.5″ S; 112° 27′ 32.9″ E). Sediments were sampled at 2–3 cm depth. Each sample comprised 10 subsamples taken in a 2-m^2 area that were mixed together. Sediment samples were freeze dried and preserved in aluminium foil at − 20 °C prior to extraction. They were then extracted and analysed in triplicate for each site.

Water was sampled at 10 cm depth in all sites using a dark, solvent-rinsed glassware, then preserved at 4 °C and transported to the laboratory for immediate processing.

Twenty-four adult specimens of *Oreochromis niloticus* (12–18 cm), the Nile tilapia, were caught from Aloo River, Surabaya River, Kalimas River and from Karangkates Dam. Nile tilapia are absent from the source of the Brantas and in the Porong River. Fish sampling followed the procedure for the use of laboratory animals for research, code of Ethics by the Committee from Brawijaya University and also followed the law on Care and Use of Laboratory Animal Resources, Nat. Res. Council, USA.

Physico-chemical analysis

Surface water physico-chemical analysis was based on procedure according to the Indonesian National Standard (SNI) for water and wastewater. Water temperature (thermometer), salinity (refractometer, Atago), pH and dissolved oxygen (DOmeter 5510, Eutech) were recorded on site at the time of sampling. Total suspended solid (TSS) was measured using the gravimetric method (SNI 06-6989.3-2004) and chemical oxygen demand (COD) was measured using the titration method (SNI 06-6989.2-2004) as described in APHA (American public health association 1992).

Sediment extraction

Sediments were extracted with dichloromethane/acetone (50/50) using an Accelerated Solvent Extraction unit (ASE 150 from Dionex). The extraction cells (10 mL) were prepared by inserting a disposable cellulose filter into the cell outlet followed by 10 g of sample. The ASE conditions were

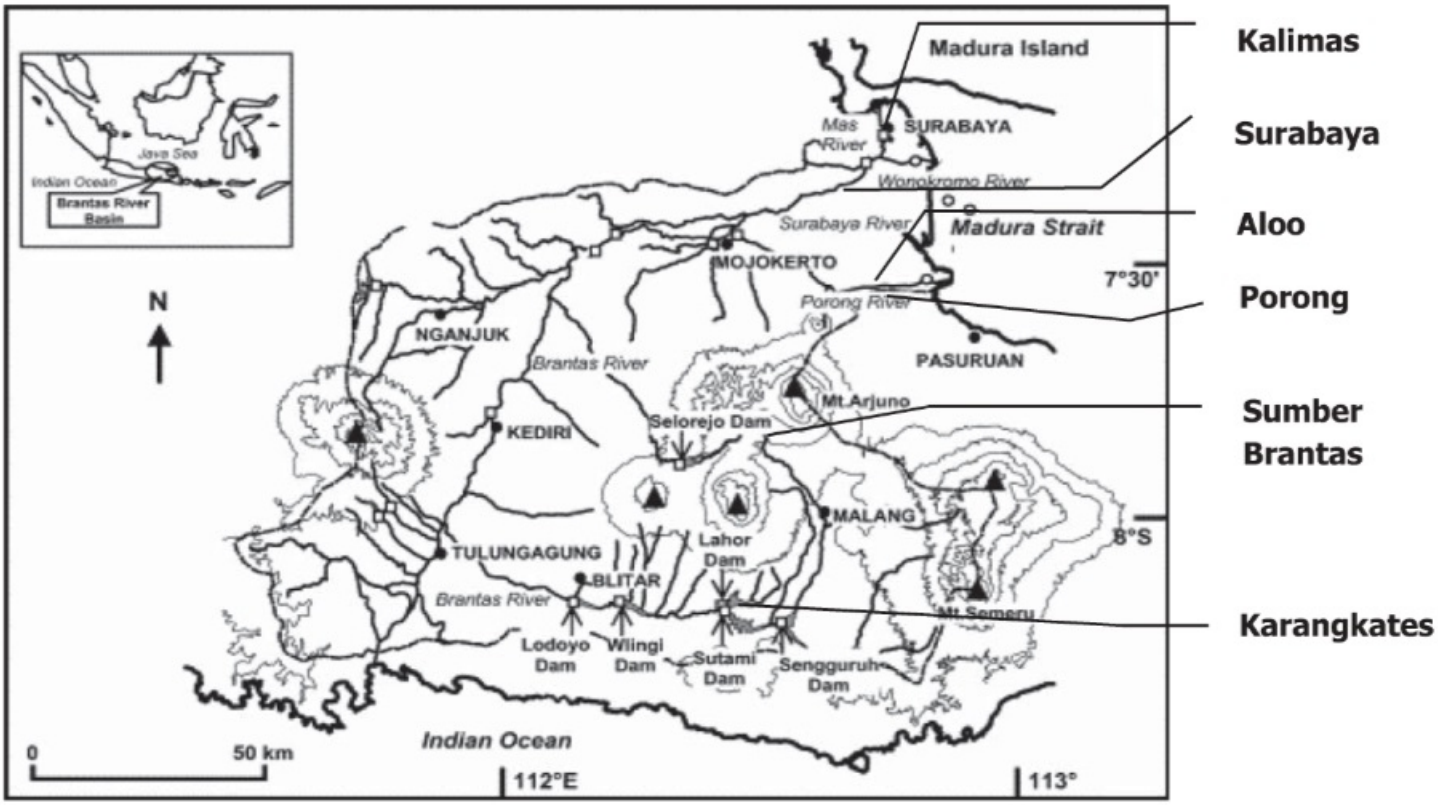

Fig. 1 Study sites along the Brantas River (scale 1:1.100.000) adapted from Balai Besar Wilayah Sungai Brantas

pressure 1500 psi, temperature 100 °C, static time 5 min and 2 cycles.

After complete evaporation of the dichloromethane, the sediment organic extracts were resuspended in 0.5 mL of dimethyl sulphoxide (DMSO, Sigma).

SOS Chromotest

The assay was carried out according to the protocol developed by Quillardet and Hofnung (1985), with and without addition of an S9 microsomal fraction obtained from the livers of Sprague-Dawley rats treated with β-naphthoflavone and phenobarbital (Xenotech). In each series of tests, a solvent control (5% v/v DMSO) and a 4-nitroquinoline N-oxide (NQO, 500 nM) or a B[a]P (benzo-a-pyrene, 6.25 μM) positive control were added. A blank of extraction was added to each series of samples, to check that the extraction procedure did not itself result in any genotoxicity. Phosphatase activity was recorded as a measure of cellular viability as described in Quillardet and Hofnung (1985). Responses were expressed as mean induction factor (IF) per gram of dw sediment for three replicates. The sample was considered as genotoxic in cases where the SOS induction factor exceeded 2, moderately genotoxic in cases where it was in the range 1.5–2.0, and not genotoxic where it was below 1.5 (Mersch-Sundermann et al. 1992).

Analyses of blood samples

Fishes were anesthetized (tricaine, MS-22, Sigma) before blood sampling. Blood samples were taken using 22-G needle attached to a sterile plastic syringe containing 50 μL of TBS (Tris Buffer Saline) from the *linea lateralis*.

Erythrocyte counts and micronuclei

To count erythrocytes and micronuclei, drops of blood were smeared on microscopic slides and allowed to dry. Peripheral blood smears were fixed in absolute methanol and kept in May Grünwalt Giemsa stain at 10%, dried out for 20 min and rinsed with distilled water. For micronuclei (MN) analysis, erythrocytes were examined using an optical microscope at × 1000 magnification. Micronuclei were characterized by its size (not more than 1/3 of the normal nuclei), without bridge linking, and not touching the main nucleus (Minissi et al. 1996). The frequency of nuclear abnormalities was recorded following the procedure of Palacio-Betancur et al. (2009).

Statistical analyses

The non-parametric Wilcoxon's two-sided test was applied to compare variations in genotoxic activity between sediment extracts. Each extract concentration was compared using the

one-way analysis of variance followed by the Dunnett's test. Homogeneity of variance was checked by Levene's test.

Analyses of variance and Tukey tests were applied to all cellular analyses (erythrocyte counts, micronuclei and other aberrations).

Differences were considered as significant if $p \leq 0.05$. All statistical analyses were performed using the SPSS software (v. 16.0, IBM).

Results

Study sites

The study was conducted in two different areas of the Brantas River, the delta and upstream sites. The sites of the delta (Porong, Aloo, Surabaya, Kalimas) are characterized by a marked reduction of dissolved oxygen, and an increase in salinity and suspended matter when compared with the upstream sites at Karangkates and Sumber Brantas (Table 1). The chemical oxygen demand was already high (22.5 mg/L) from the Karangkates site as the river had passed through the cities of Batu and Malang.

Genotoxicity of sediment extracts

The crude organic extracts from sediments were analysed using the SOS Chromotest. Except for the extract from the Kalimas River, no significant genotoxicity could be measured in any activated (with S9 microsomal fraction) sediment extracts (Fig. 2). Only for the highest dose tested, an elevated, but low, induction factor (IF = 1.2) could be seen in the extract from the Kalimas River when compared with the control. Even when the extracts were first activated with S9 microsomal fraction, no genotoxicity could be assessed for sediment extracts from sites located upstream at Sumber Brantas and at the Karangkates reservoir (Fig. 2). However, clear dose-responses were observed for all crude sediment extracts from the Brantas Delta (Porong, Aloo, Kalimas and Surabaya rivers), clearly suggesting the occurrence of genotoxic compounds in the organic extracts. The calculated SOS induction factors were above 1.5 for the Aloo (both for the medium and maximum dose) and the Surabaya rivers (only at the maximum dose tested), indicating that these sediments can be stated as moderately genotoxic. The induction factor, although elevated, remained below this threshold for the Porong and Kalimas rivers.

Micronuclei in erythrocytes of Nile tilapia

Blood samples from Nile tilapia inhabiting the rivers of the Brantas catchment were analysed. Erythrocytes were visualized and counted under microscope. Interestingly, there were significant variations of cell density according to sampling sites (Fig. 3). Number of blood cells in fishes caught in the upper site of the Brantas (Karangkates) were 2.5 million/mL and higher than at the other sites. The cell concentration was significantly reduced by 50 and 42%, respectively, in fish caught in the Surabaya and the Kalimas rivers.

The number of micronuclei was particularly high in all blood samples collected. They represented nearly 2% of the studied cells at Karangkates, the site located upstream of the Brantas River. However, the number of cells displaying micronuclei was even more numerous in blood samples from fish collected in the Brantas delta, with percentages above 3% of the observed erythrocytes (Fig. 4).

The observation of erythrocytes led to the discovery of numerous cytoplasmic vacuolizations devoid of staining and of basophilic granulations on the periphery of the nuclei (Fig. 5). Although further study is required to clearly identify these chromogenic changes, they might correspond to lipofuscin-like and cerebroid-like material that are accumulated in cells. These "aberrations" have been counted and results indicate that they were significantly more numerous in erythrocytes from fish caught in the rivers of the delta than those from the upstream site at Karangkates (Fig. 6).

Discussion

The aim of this study was to investigate the putative occurrence and subsequent effects of genotoxic compounds in

Table 1 Water quality data of sampling sites. *DO*, dissolved oxygen; *TSS*, total suspended solid; *COD*, chemical oxygen demand. *Standard as stated in the government regulation of Republic of Indonesia (PP no. 82, 2001, Indonesia 2001). #Data from Roosmini et al. 2018, Rahadi et al. 2018

Parameters	Sumber Brantas#	Karangkates	Aloo	Porong	Surabaya	Kalimas	Water quality standard *
DO (mg/L)	7.2	5.6	2.6	3	4	3.5	>3
pH	-	7.5	8.0	7.0	7.0	7.0	6–9
Salinity (ppt)	0	0	5	5	4	2.7	-
TSS (mg/L)	7.5	16.8	131.7	330	213.7	223	50
COD (mg/L)	8.5	22.5	18.8	20.1	14.1	13.8	25

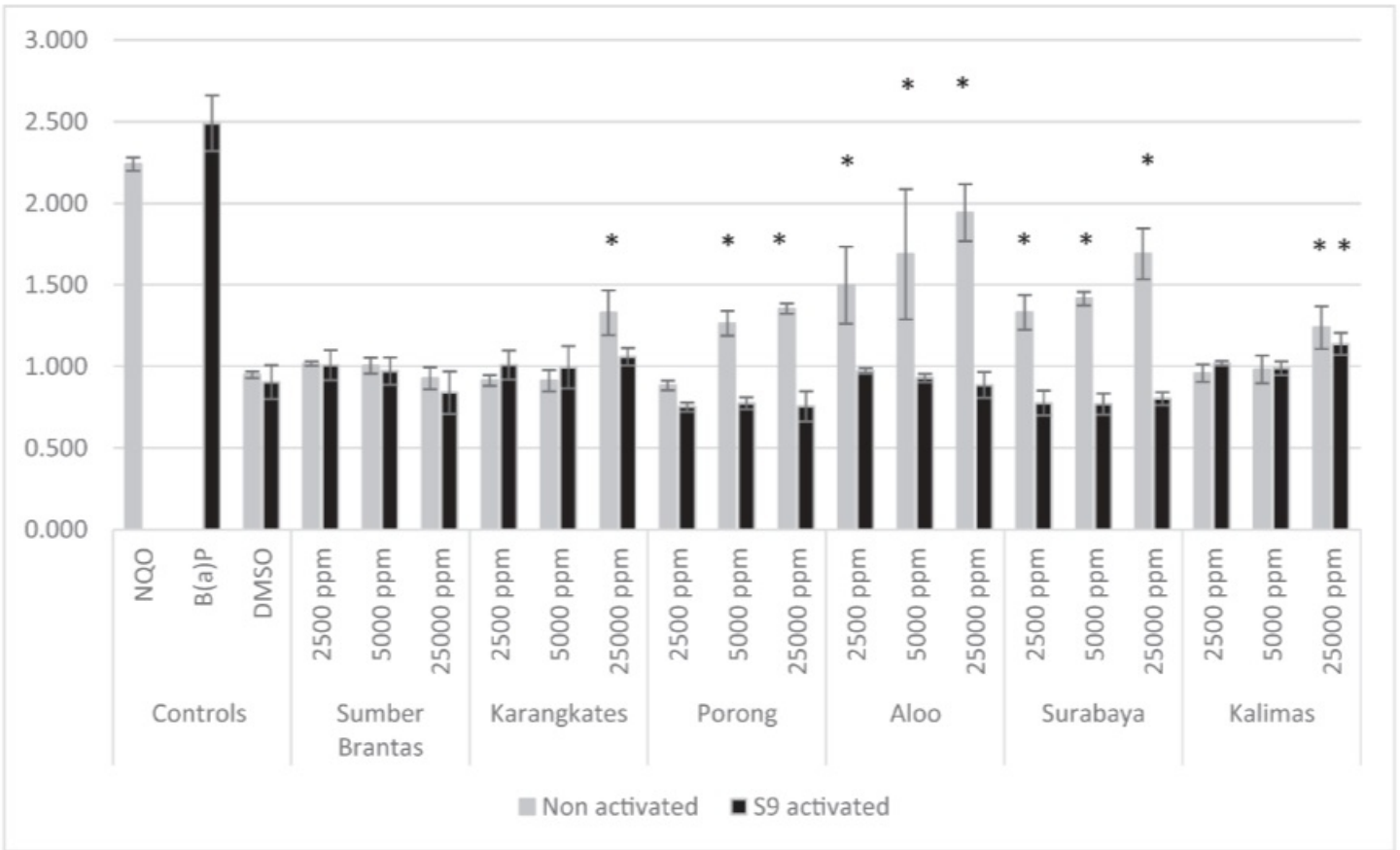

Fig. 2 Genotoxicity of sediment extracts from the Brantas River as measured using the SOS Chromotest with or without prior activation (S9). Three doses were tested for each sediment extract. NQO and B(a)P are positive controls. Bars represent mean ± SD ($n = 3$). An asterisk indicates significant differences from the DMSO control ($p < 0.05$)

Indonesian rivers of East Java in the Brantas catchment. The results show that the sites in the delta have suspended solids concentrations that far exceed Indonesian water quality standards (Indonesia 2001). These sites, particularly Surabaya and Kalimas Rivers, are polluted by domestic and industrial wastes. Moreover, the Aloo River and the Porong River are under the influence of the LUSI volcano mudflow since 2006 (Davies et al. 2008). Conversely, the upstream part of the Brantas is moderately polluted (Roosmini et al. 2018). The SOS Chromotest was used to assess the presence of active compounds in sediments sampled along the Brantas River from its source to the delta. Crude organic extracts did not

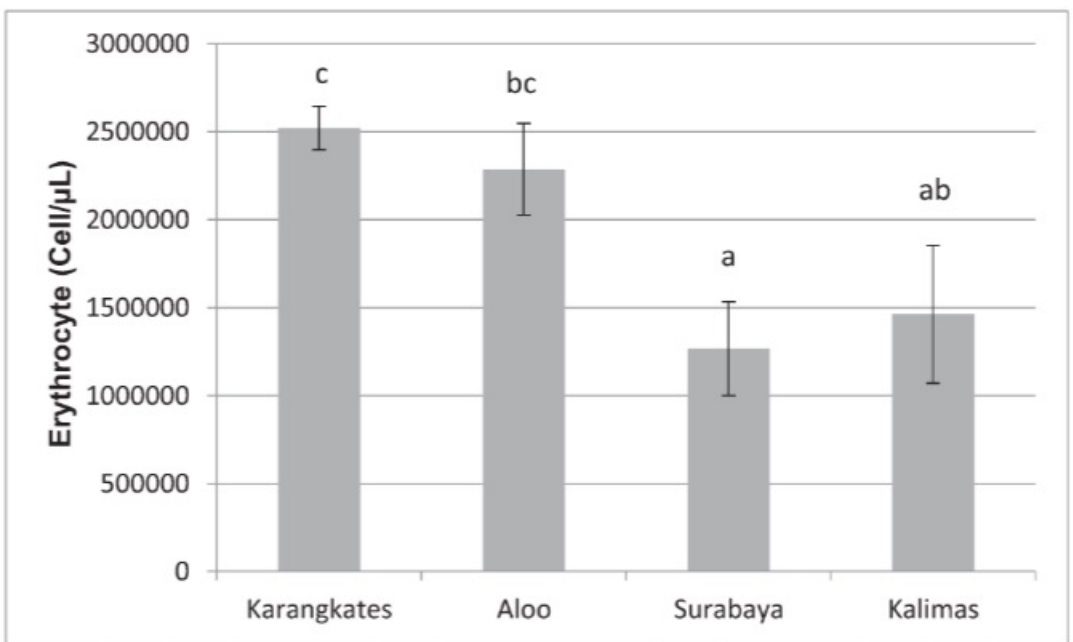

Fig. 3 Erythrocyte number in *Oreochromis niloticus* caught from different sites in the Brantas catchment. Bars represent mean ± SD ($n = 4$); letters indicate significant differences ($p < 0.05$)

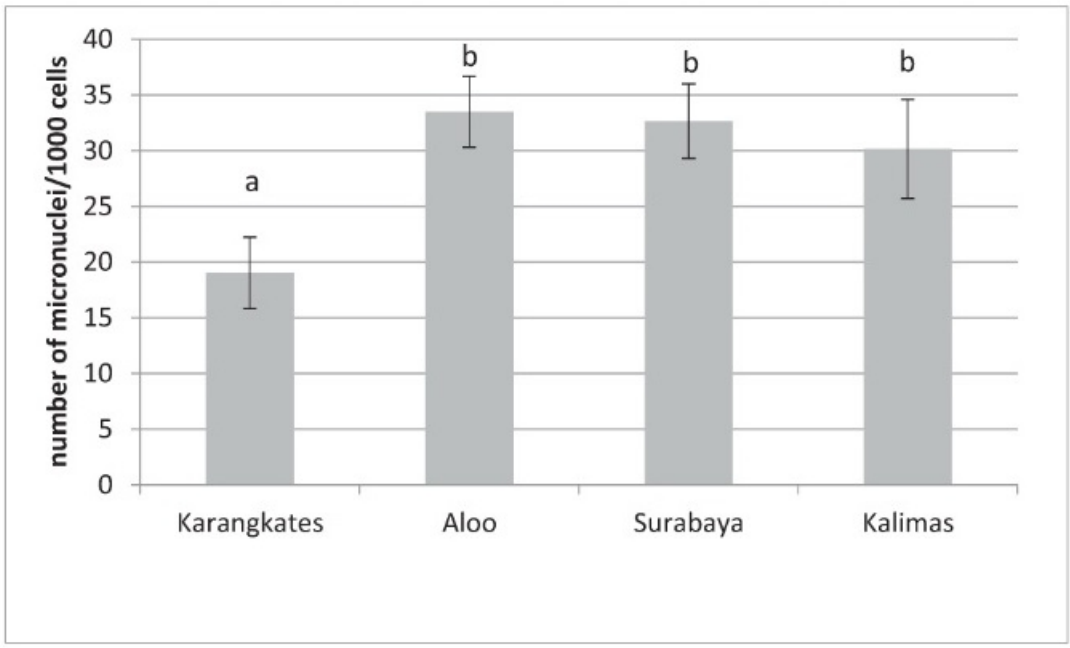

Fig. 4 Number of micronuclei in *Oreochromis niloticus* erythrocytes caught from Karangkates Dam at the upstream part of the Brantas and from rivers at the downstream part of the Brantas (Aloo, Surabaya and Kalimas). Bar represents mean ± SD ($n = 4$). Letters indicate significant differences ($p < 0.05$)

show any genotoxicity in samples from the upper part of the river. On the contrary, all sediment extracts from the four investigated rivers in the Brantas Delta induced a dose-dependent increase in response indicative of genotoxicity. The measured induction factors for the extracts from the Surabaya River and from the Aloo River were higher than the threshold of 1.5 per gram of sediment, indicating moderate genotoxicity (Mersch-Sundermann et al. 1992). These results were obtained without any incubation with S9 microsomal fraction, thus advocating for the presence of direct genotoxic compounds in the extracts. When tested after S9 activation, only the sediment extract from the Kalimas River showed little (below the threshold of 1.5) but significant induction of the SOS response.

High levels of assessed genotoxicity were reported from studies on polluted rivers in other parts of the world. For example, the SOS induction factors obtained for the Seine estuary (France) ranged from 0.80 to 9 per gram of dw sediment (Cachot et al. 2006). In the St. Lawrence River (Canada), tests on sediment extracted resulted in an induction factor in the range of 1–2 (Langevin et al. 1992; White et al. 1998). In the case of the sediments from the Seine and the St Lawrence

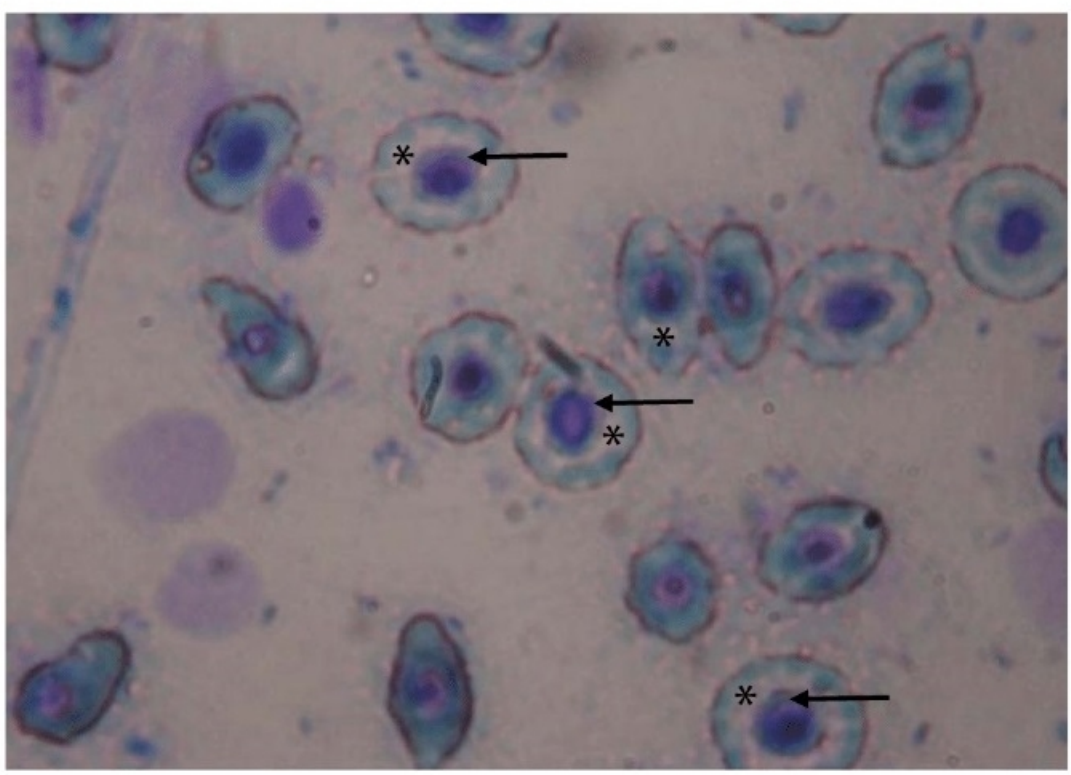

Fig. 5 *Oreochromis niloticus* erythrocytes showing cytoplasmic vacuole-like formations (asterisks) devoid of staining and chromogenic lipofuscin-like accumulations in the periphery of the nucleus (arrows)

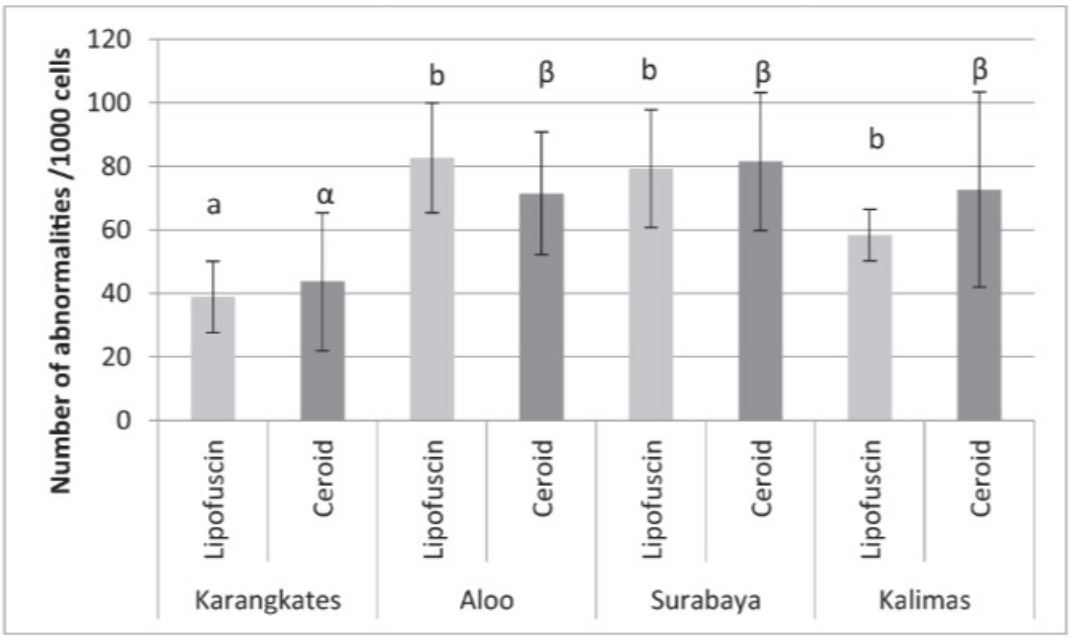

Fig. 6 Lipofuscin-like and ceroid-like structures in erythrocyte cells of *Oreochromis niloticus* caught at Karangkates in the upper part of the Brantas and from rivers downstream of the Brantas catchment (Aloo, Surabaya and Kalimas). Bars represent mean ± SD ($n = 4$); letters indicate significant differences ($p < 0.05$)

river, the genotoxicants were only pro-genotoxic. Cachot et al. (2006) identified these compounds mainly as high molecular-weight polycyclic hydrocarbons and, to a lesser extent, as unknown polar organic compounds. This could show that rivers from the Brantas delta are less contaminated by industrial compounds including hydrocarbons. Indeed, this was also the result of a comparison of water quality of Japanese rivers and Indonesian rivers. Although the Indonesian rivers were heavily contaminated by domestic sewage, from the view point of industrial pollution, water of rivers in Indonesia was not so heavily polluted as that in Japan (Kido et al. 2009). Both direct-acting and pro-genotoxic substances have been identified in bivalve molluscs from the Saguenay Fjord (Canada). In this study, a significant relationship between a demographic variable population near shoreline and genotoxic potency suggests that the accumulated direct-acting genotoxins may be of municipal origin (White et al. 1997). A similar origin of the direct-acting genotoxicants, deriving from sewage waste, cannot be ruled out for the Brantas River.

In vivo genotoxic effects were looked for in fish inhabiting the Indonesian rivers. Occurrence of micronuclei in erythrocytes was very high in all investigated sites, accounting for nearly 2 to 3% of the fish blood cells. This occurrence was more pronounced and significantly elevated in the downstream sites of the Brantas delta. The most impacted site regarding this biological indicator was the Aloo River which is also the one that lead to the identification of the highest sediment genotoxicity using the SOS Chromotest. It is noteworthy that this site is under the influence of volcano mud effluents characterized by high amounts of metals including Mn, Zn, Cu, Cr, Cd, Pb, Co, Ni, Hg and As (Krisnayanti and Agustawijaya 2014). However, identity of the compounds responsible for the occurrence of micronuclei cannot be stated from this study. Many compounds, including organic contaminants have been shown to induce these formations (Bolognesi et al. 2006). High levels of micronuclei (up to 1.8%) have been also reported in Nile tilapia in Egypt (Omar et al. 2012). Similar percentage of micronuclei have been reported in the Aloo River and the Brantas in a related species, *Oreochromis mossambicus* (Muhusini 2011). This suggests both a high sensitivity of both species and the occurrence of potent genotoxic compounds in the investigated Indonesian rivers.

Microscopic observations revealed that erythrocyte concentration differed according to sampling site. Lower concentrations were counted in downstream sites compared with the upstream sites of the Brantas River. The reduction of erythrocyte cells in *O. niloticus* at Surabaya and Kalimas River could be explained by the possible decrease of health and immune system as shown in our previous study for *Oreochromis mossambicus*, *Chanos chanos* and *Channa striata* from the same sites location (Risjani et al. 2014) or by haematopoiesis alteration caused by water pollutants (Zaghloul et al. 2007). No sign of internal haemorrhaging nor signs of parasites was apparent upon dissection of the fish. Moreover, erythrocytes from the Brantas delta showed evidence for higher occurrence of cellular alterations. Compared with the fish blood cells sampled in the sites located in the upper part of the Brantas River, the erythrocytes from fish caught in the Brantas Delta had a higher number in cytoplasmic vacuolizations and of basophilic granulations on the periphery of the nuclei. Although this would need further explorations to be confirmed, these alterations could correspond to lipofuscin accumulation as a consequence of oxidative stress. In line with our study, work conducted by Çavaş and Ergene-Gözükara (2005) showed that the frequencies of both micronuclei and other nuclear abnormalities in grey mullets (*Mugil cephalus*) captured from polluted areas were significantly higher than those

in mullets from the reference area. These increased associations of micronuclei and cellular aberrations have also been reported in Nile tilapia in Egypt (Omar et al. 2012) or after experimental exposure to 1-nitropyrene (Bacolod et al. 2017).

In conclusion, this study showed (1) evidence for the occurrence of genotoxic compounds in sediments from rivers of the Brantas Delta and especially in the Aloo River based on the SOS Chromotest results, (2) high numbers of micronuclei in erythrocytes of Nile tilapia caught in all investigated rivers and (3) cytoplasmic alterations which could be indicative of the presence of lipofuscin and thus of the occurrence of oxidative stress in the cells. Altogether, this draws a picture that indicates that the Brantas catchment and to a higher extent the downstream sites are contaminated by genotoxic compounds that can alter the health of the fish. The sites under the influence of volcano mud may be especially at risk. Our data suggest that sediments from these rivers may constitute a major source of pollution and hazards for species living or feeding in the area.

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