Article

The Effect of Humic Acids on the Phototoxicity of Benzo[a]pyrene to the Alga, *Pseudokirchneriella subcapitata*

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Abstract

To evaluate the binding affinity of benzo[a]pyrene (BaP) and the detoxification effect of humic acids (HAs) on the phototoxicity of BaP, we conducted measurements of the partition coefficients (K_{oc}) and carried out BaP phototoxicity tests using the green alga, *Pseudokirchneriella subcapitata*, with six HAs derived from three different soil types. The K_{oc} values for Andosols HAs were higher (15-29 × 10^6 L kg C^{-1}) than those for Cambisols HAs and Histosols HAs. A correlation between K_{oc} values and the aromatic moiety was found, however Histosols HAs tended to deviate slightly from this trend. Growth inhibition of *P. subcapitata* by BaP decreased with the addition of all tested HAs. Andosols HAs in particular showed a strong detoxifying effect on the phototoxicity of BaP. Although no clear relationships between the structural properties of the six HAs and the detoxifying effect were found, it was inferred that the binding of BaP to HA is involved in the mechanism of reducing BaP toxicity to the alga. Restoration of the growth inhibition ratio in the presence of Histosols HAs occurred at a higher HA concentration range. We conclude that due to higher photosensitivity, Histosols HAs generated a highly toxic substance from the interaction with BaP, resulting in differences to the effects of the mineral soil-derived Cambisols and Andosols HAs.

Keywords: Humic Acid, Benzo[a]pyrene, Phototoxicity, Binding Affinity, Polycyclic Aromatic Hydrocarbon, Detoxification

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are some of the most widespread environmental pollutants. They consist of two or more fused aromatic rings and have toxic, carcinogenic, and mutagenic effects. PAHs are mainly formed anthropogenically, by pyrogenic process like the incomplete combustion of fossil fuels and biomass, and petrogenic process like the transportation, storage, refine and use of petroleum products. However they are also formed through natural biological processes in terrestrial and marine environments (Abdel-Shafy and Mansour, 2016).

Benzo[a]pyrene (BaP) is a representative PAH. It features five aromatic rings, and is widely found in soil, sediment, water and air. It was the first PAH isolated and was identified as the major cancer-producing compound of coal-tar (Cook et al., 1933). Owing to its high octanol-water partitioning coefficient (K_{ow} = 6.13) and high molecular weight (Mw 252.32), it is recalcitrant to microbial degradation and persists in soil for decades (Lily et al., 2009; Hernández-Castellanos et al., 2013). Although no epidemiological data on BaP alone are available, this compound has been classified as a human carcinogen (Group 1) in the International Agency for Research on Cancer (IARC) monograph (2012). The United States Environmental Protection Agency has classified it as one of the 16 PAHs in the list of priority pollutants (2008), and has characterized it as carcinogenic to humans in the Integrated Risk Information Systems (IRIS) database (2017). In Japan, BaP has also been defined as a compound requiring priority action with regard to environmental issues (Air Pollution Control Act; Environmental Risk Assessment Office [ERAO], Ministry of the Environment, Japan,

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2006). In addition to carcinogenicity, BaP has been demonstrated to cause developmental, reproductive, and immunotoxicity in mammals (IRIS, 2017). It has also been reported that BaP shows acute toxicity and/or bioaccumulation in aquatic organisms (ERAO, 2006; Sushkova et al., 2018).

Natural organic materials and in particular humic substances (HS), which comprise a major fraction of natural organic matter in soil, water and sediments, play an important role in sorption, desorption and decomposition processes involving PAHs. They have been shown to exert great influence on the fate and distribution of PAHs in the environment (Huang and Weber, 1997; Kleineidam et al., 2002; Ahangar, 2010), and greatly affect their toxicity and bioavailability (Lueking et al., 2000). The hydrophobicity of BaP and the structural properties of HS, such as aromaticity and atomic H/C ratio, are involved in the binding of BaP to HS (McCarthy and Jimenez, 1985a; Paolis and Kukkonen, 1997; Kohl and Rice, 1998; Kögel-Knabner et al., 2000; Steinberg et al., 2000; Conte et al, 2001; Kollist-Siigur et al., 2001; Saparpakorn et al., 2007; Zhang et al., 2009a, 2009b), and it has also been reported that both structure and source of HS exert a reduction effect on the bioaccumulation (McCarthy and Jimenez, 1985b, McCarthy et al., 1985; Kukkonen and Oikari, 1987; Haitzer et al., 1999a; Steinberg et al., 2000) and the mutagenic effects of BaP (Sato et al., 1986; 1987a, b). Yet despite considerable concerns about BaP toxicity and environmental impacts, its interactions with HS remain much less studied than those of other PAHs (e.g. phenanthrene, anthracene, and pyrene). It is known that BaP toxicity in aquatic organisms is enhanced by light irradiation (Cody et al., 1984; Schoeny et al., 1988) and that HS is also photochemically changed (Frimmel, 1994; Moran and Zepp, 1997; Aguer et al., 1999; Lou and Xie, 2006). However, only a few studies report on the interaction between HS and BaP phototoxicity (Gensemer et al., 1998), and these reports do not take a variety of HS into account. This information is essential for predicting the toxic behaviour of BaP in the environment.

The present study aims to evaluate the binding affinity of BaP and the detoxification effect of HS on its phototoxicity in consideration of their structural variety. Humic acids (HAs) are the major components of HS and show unique structural properties connected to their origins. In particular, the structural properties of soil HAs differ markedly with their origins compared to soil fulvic acids and aquatic HS (Malcolm, 1990). Soil HAs also have the advantage of being easy to obtain in relatively large quantities. We therefore employed soil HAs in the first step of the present study. HAs derived from three different soil types were used to measure partition coefficients (K_{oc}) and applied to a BaP phototoxicity test using a green alga, *Pseudokirchneriella subcapitata*.

Materials and Methods

Chemicals

Benzo[a]pyrene (purity 97%+, water solubility 1.61×10^{-3} mg kg^{-1}) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Concentrated stock solution (0.1 mg L^{-1}) in acetone was used for sorption experiments, and stock solution (0.025 mg L^{-1}) in ethylene glycol monoethyl ether (EGME) was used for acute toxicity tests. An aliquot of BaP stock solution was diluted with EGME, such that the desired concentrations in algal cultures could be achieved by addition of 0.1 ml. All other chemicals were of analytical grade.

Preparation of stock solution of HAs

The HAs used in this study were prepared from the A horizon of two Cambisols (Hanaore, HO; Nishiotafuku, NOF), two Histosols (Ichijima, IJ; Tomintoul, TMT), and two Andosols (Nagao, NGO; Sugadaira, SG) samples according to the methods described by the International Humic Substances Society (Swift, 1996). Descriptions of the soil samples are shown in Table 1. The HAs were dissolved in 0.1 mol L^{-1} NaOH, diluted with MQ water, and then adjusted to pH 7.5 with 0.1 mol L^{-1} HCl. Concentrated stock solution of HAs (approximately 2 g L^{-1}) was sterilized by membrane filtration (DISMIC 25CS, pore size 0.2 μm; Advantec Toyo Kaisha, Tokyo, Japan).

Characterization of HAs

The amount of carbon, nitrogen, and hydrogen in HAs was determined using an elemental analyser (2400-C; Perkin Elmer Japan, Yokohama, Japan). The oxygen content (%) was derived using the formula [100 - (C% + H% + N%)]. The ash content was determined by combustion at 550°C. All results were expressed on a moisture- and ash-free basis. Solution ^{13}C NMR spectra were obtained on a Bruker Avance

Table 1 Descriptions of soil samples used for extraction of HAs.

HA	Soil type	Soil name	Location (Prefecture or council, Country)	Land use
HO	Cambisols	Hanaore	Hyogo, Japan	Forest
NOF	Cambisols	Nishiotafuku	Hyogo, Japan	Forest
IJ	Histosols	ichijima	Hyogo, Japan	Grassland
TMT	Histosols	Tomintoul	Moray, Scotland, UK	Grassland
NGO	Andosols	Nagao	Miyazaki, Japan	Forest
SG	Andosols	Sugadaira	Nagano, Japan	Grassland

500 K spectrometer (Bruker, Karlsruhe, Germany) using sample tubes 5 mm in diameter, according to the method described by Yanagi et al. (2011). Chemical shifts were referenced with an internal standard of sodium 3-trimethylsilylpropionate-2,2,3,3,D4 (Euriso-top, Saint Aubin, France). Aromaticity was calculated following Hatcher et al. (1981). Content of phenolic hydroxyl Groups was determined colorimetrically with Folin-Ciocalteu’s reagent (Tsutsuki and Kuwatsuka, 1978). Gallic acid was used as the calibration standard sample.

Sorption experiments

A fluorescence quenching technique was used according to Gauthier et al. (1986) to determine the K_{oc} between BaP and HAs. BaP stock solution in acetone (final conc. 1.0 μg L^{-1}) was added to the 13-mL glass vial and the acetone was evaporated. 5 mL of carbonate buffer (pH 7.5) was then added to the vial, which was shaken for 24 h to dissolve the BaP in the buffer. Five mL of HA solution diluted with the carbonate buffer in final concentrations ranging from 0 to 2.6 mg C L^{-1} was added to the vial and shaken for 24 h to bind BaP with HAs. The quenching of BaP fluorescence was measured using a spectrofluorometer (FP-8200; JASCO, Tokyo, Japan) at 25 °C. The excitation and emission wavelengths were 380 nm and 405 nm respectively (McCarthy and Jimenez, 1985a) with a bandwidth of 5 nm. The fluorescence intensity of BaP was corrected for the influence of background HA and inner filter effect. The corrected fluorescence intensity of BaP with (F) /without (F_0) HA was used in the Stern-Volmer equation. K_{oc} values were calculated from the slopes of the Stern-Volmer plots. Three replicates were prepared for each type and concentration of HA.

Phototoxicity tests

Toxicity tests were performed on the basis of the modified OECD standard procedure (OECD, 2006) and conducted in a series of 50-mL flasks. 10 mL of NIES-C medium (pH 7.5; Ichimura, 1971) containing HA solution in concentrations ranging from 0 to 1.1 mg C L^{-1} was added to the test flask. BaP stock solution in EGME was added to this flask to achieve a final concentration of 5 μg L^{-1} and shaken for 24 h at 25 °C to bind BaP with HA. The green alga, *P. subcapitata* (strain NIES 35), obtained from the National Institute for Environmental Studies (NIES; Tsukuba, Japan), was used as a test organism and was cultured in NIES-C medium at 25 °C with a 16:8 light/dark photoperiod. Inoculate was taken from the exponentially growing pre-cultures and inoculated into the test flask at a density of 10^4 cells ml^{-1}. Three replicates were prepared for each HA type and concentration. Cultures containing EGME instead of BaP were prepared as a control for each HA concentration. Each measurement was also taken in untreatment (absence of HA and BaP) and treatments containing only BaP (absence of HA). All flasks were incubated at 25 °C in a shaking incubator (130 rpm) with a 16:8 light/dark photoperiod. After 96 h incubation, algal cell concentrations were determined using a hemocytometer. At each HA concentration, the relative growth ratio of *P. subcapitata* in the control was expressed as a percentage of untreatment, while the growth inhibition ratio by BaP was expressed as a percentage of control.

Results

Properties of HAs

The elemental compositions and degree of unsaturation of the six tested HAs are shown in Table 2. The carbon and oxygen contents ranged from 53.3 % for NOF to 58.7 % for IJ and 32.1 % for HO to 38.5 % for NGO, respectively. The hydrogen and nitrogen contents of Cambisols HAs showed the highest values, whereas the lowest values were present in Andosols and Histosols HAs, respectively. The H/C atomic ratio ranged from 0.79 to 1.28, with the highest ratio being present in Cambisols HAs, followed by Histosols and Andosols HAs. In contrast, the O/H atomic ratio, ranging from 0.33 to 0.61, showed the highest values in the reverse order of H/C ratio. The degree of unsaturation, calculated following Tsutsuki and Kumada (1980), ranged from 39.2% for HO to 62.9 % for SG. These values, representing the chemical properties of HAs, were distributed widely across the range commonly reported for soil HAs in previous studies (Schnitzer and Khan, 1972; Kuwatsuka et al., 1978; Steelink, 1985; Kumada, 1987; Yonebayashi and Hattori, 1988; Rice and McCarthy, 1991). The Ash contents ranged from 1.4 % for IJ to 5.4 % for SG.

The composition of carbon functional Groups and the aromaticity of HAs calculated from ^{13}C NMR spectra are listed in Table 3. The percentage of alkyl carbons ranged from 6.4% for SG to 24.8% for HO; that of aryl carbons ranged from 22.8% for NOF to 52.9% for SG; and that of O-alkyl, phenolic, carboxylic, and carbonyl carbons from 11.6% to 30.3%, 5.3% to 11.4%, 10.9% to 21.5%, and 1.5% to 6.8%, respectively. SG had the lowest alkyl and O-alkyl carbons and the highest aryl carbons, as well as the highest aromaticity. Although the carbon distribution of NGO derived from Andosols at a forest locality was similar to that of SG, the percentages of alkyl and O-alkyl carbons were relatively higher, and the percentages of aryl carbons and aromaticity were lower. The Cambisols HAs (HO and NOF) showed the highest alkyl carbon contents as well as the lowest aryl carbon contents and aromaticities compared with other HAs. Both Histosols HAs had higher phenolic carbon contents (11.4% for IJ and 10.0% for TMT) than other HAs, but differed amongst each other in aryl and carboxylic carbon contents. Polyphenol contents are also shown in Table 3. The Histosols HAs had the highest contents (16.7 g kg^{-1} for IJ and 19.4 g kg^{-1} for TMT) followed in order by Cambisols and Andosols HAs.

Partition coefficient of BaP to HAs

Stern-Volmer plots for fluorescence quenching of BaP with six HAs are shown in Figure 1. Linearity of the plots was observed for all HAs used in this study, and F_0/F increased with HA concentration. Among the six HAs, SG showed the highest affinity to BaP, followed by NGO. Thus, Andosols HAs were found to have the highest affinity among the tested HAs derived from three types of soil. On the other hand, HO derived from Cambisols HAs showed the lowest affinity, and IJ and TMT derived from Histosols HAs had similar values. Consequently, SG and NGO

Table 2 Elemental composition and atomic ratios of the six HAs.

HA	**Element content** (weight %)				**Atomic ratio**				DU*	Ash (%)
	C	**H**	**N**	**O**	**H/C**	**O/C**	**N/C**	**O/H**		
HO	57.2	6.1	4.6	32.1	1.28	0.42	0.07	0.33	39.2	5.2
NOF	53.3	5.2	4.3	37.2	1.18	0.52	0.07	0.45	44.6	3.3
IJ	58.7	4.7	2.0	34.6	0.96	0.44	0.03	0.46	53.3	1.4
TMT	56.2	4.5	1.7	37.6	0.96	0.50	0.03	0.52	53.5	3.4
NGO	53.6	4.1	3.7	38.5	0.92	0.54	0.06	0.58	56.8	2.9
SG	56.4	3.7	3.4	36.5	0.79	0.49	0.05	0.61	62.9	5.4

*DU: Degree of unsaturation was calculated using (2C + N - H) / 2C × 100 from elemental composition (atomic %).

Table 3 Composition of carbon functional groups, aromaticity and polyphenol content.

HA	**% of carbon species (chemical shift, δ ppm)**						Aromaticity	**Polyphenol content** (g kg^{-1})
	Alkyl (5-48)	**O-Alkyl** (48-110)	**Aryl** (110-145)	**Phenolic** (145-165)	**Carboxylic** (165-190)	**Carbonyl** (190-230)		
HO	24.8	25.7	23.5	5.3	17.7	3.0	0.36	9.8
NOF	22.2	30.3	22.8	6.1	15.6	3.0	0.35	9.2
IJ	17.7	16.1	42.4	11.4	10.9	1.5	0.61	16.7
TMT	11.7	16.7	33.2	10.0	21.5	6.8	0.60	19.4
NGO	11.7	23.3	33.8	7.7	18.5	4.9	0.54	7.8
SG	6.4	11.6	52.9	5.3	19.6	4.2	0.76	7.3

showed high K_{oc} values, calculated from the slope of the Stern-Volmer plots, at 28.95 and 14.98 × 10^6 L kg C^{-1}, respectively (Table 4). NOF showed a lower K_{oc} value of 4.67 × 10^6 L kg C^{-1}, and HO, IJ, and TMT showed values of 1.7 to 1.87 ×10^6 L kg C^{-1}.

Growth of **P. subcapitata** *in the presence or absence of BaP and HAs*

The relationships between the relative growth ratio of *P. subcapitata* in the absence of BaP and the concentration of HAs are shown in Figure 2. Ratios fluctuated to different degrees at every HA concentration, and there was no relationship between relative growth ratio and HA concentration.

The relationships between ratio of growth inhibition of *P. subcapitata* by BaP and the concentration of HAs are shown in Figure 3. Ratios decreased with the addition of all the HAs used in this study. SG showed

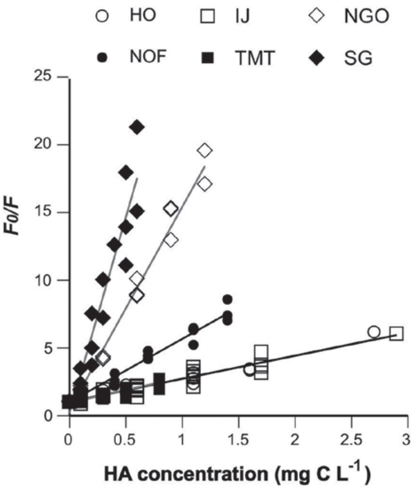

Figure 1. Stern-Volmer plots for fluorescence quenching of BaP with six HAs. Solid lines indicate the results of linear regression analysis.

Table 4 The K_{oc} values and the correlation coefficient of BaP for six HAs.

HA	**K_{oc}** (×10^6 L kg^{-1})	***r***	**Log K_{oc}**	**Median mitigating concentration** (mg C/L^{-1})
HO	1.70	0.977	6.23	1.23
NOF	4.67	0.984	6.67	1.05
IJ	1.75	0.963	6.24	0.81
TMT	1.87	0.919	6.27	1.90
NGO	14.98	0.991	7.18	0.51
SG	28.95	0.952	7.46	0.32

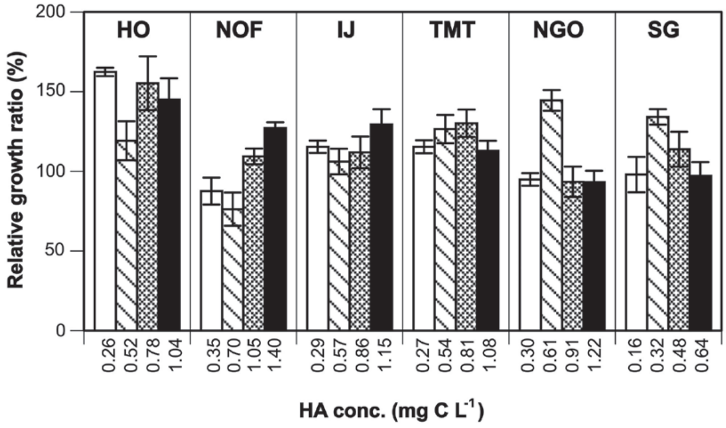

Figure 2. The relationships between the relative growth ratio of *P. subcapitata* in the absence of BaP and the concentration of HAs.

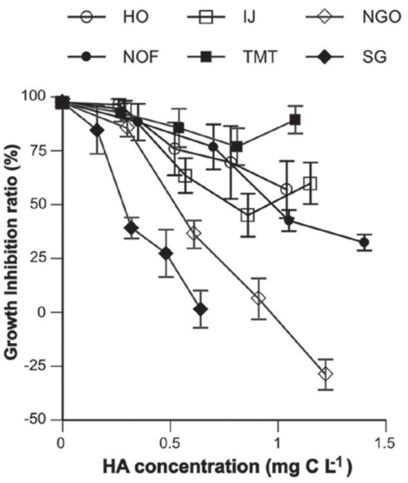

Figure 3. The relationships between the growth inhibition ratio of *P. subcapitata* by BaP and the concentration of HAs.

the strongest mitigation of growth inhibition by BaP among the six HAs, reducing inhibition by almost 100%. The growth inhibition ratio of NGO was -28.9% at the maximum HA concentration (1.22 mg C L^{-1}), indicating growth promotion. The two Cambisols HAs (HO and NOF) showed moderate mitigation effects. Although growth inhibition ratios decreased with increasing HA concentrations in these four HAs, those of IJ and TMT, derived from Histosols HAs, increased at the maximum concentration (IJ: 59.9%, TMT: 89.4%). The HA concentration that mitigated growth inhibition of *P. subcapitata* by 50% (median mitigating concentration: MMC), calculated from the slope of a linear approximation of each ratio series (except for the highest concentration of Histosols HAs), showed a minimum value of 0.32 mg C L^{-1} for SG and a maximum value of 1.90 mg C L^{-1} for TMT (Table 4). Andosols HAs and Cambisols HAs tended to show strong and weak mitigating effects at low concentrations, respectively. In contrast, the detoxifying effect for Histosols HAs showed a large difference between the two HAs.

Discussion

Binding affinity of BaP to HAs

All HAs used in this study showed a binding affinity to BaP. The K_{oc} and logK_{oc} values calculated from the Stern-Volmer plot (Table 4) were nearly equal to (McCarthy and Jimenez, 1985b; McCarthy et al., 1989; Döring and Marschner, 1998) or higher (McCarthy et al., 1989; Paolis and Kukkonen, 1997; Haitzer et al., 1999a; Zhang et al., 2009a, 2009b) than those found in previous reports. Most of these previous studies used aqueous HS (water- or sediment-derived). It has been reported that aqueous HAs show fairly low K_{oc} values relative to other PAHs, in comparison with HAs derived from soil (Gauthier et al., 1987; Chin et al., 1997). K_{oc} values were also found to increase with decreasing BaP concentrations (Zang et al., 2009a, 2009b). The BaP concentration used in this study (1 μg BaP L^{-1}) was lower than most of those previously studied. It can therefore be concluded that the K_{oc} values in our study had the relatively highest values.

The HAs used in this study showed K_{oc} values in the following descending order: Andosols >> Cambisols ≈ Histosols. Perminova et al. (1999) examined the binding affinities of HS from various origins to three PAHs (anthracene, pyrene, and phenanthrene). They found a significantly higher K_{oc} value for chernozemic HA than for gray wooded soil HA and peat HS. Since chernozemic HAs, characterized by high aromaticity, exhibit very similar structural properties to Andosols HAs as reported by Fujitake et al. (2010) and Tani et al. (2012), our results are consistent with the findings of these authors.

The relationship between the main structural properties of HAs and the K_{oc} values obtained in this study was examined (Table 5). The only correlation found was with the O/H ratio and ∑CAr/∑CAlk [(aryl carbons + phenolic carbons) / (alkyl carbons + O-alkyl carbons)], calculated from the carbon distribution by way of ^{13}C NMR spectra, which represents the prevalence of an aromatic core over aliphatic periphery or lipophilic-lipophobic balance of the molecule (Perminova et al., 1999). Similarly, the correlation between the K_{oc} values of PAHs to HS and the aromatic carbon content of HS (including atomic H/C ratio, absorptivity at 280 nm, and ΣCAr/ΣCAlk) has been reported in several studies (Gauthier et al., 1987; Chin et al., 1997; Perminova et al., 1999; Steinberg et al., 2000; Kobayashi and Sumida, 2015). This may be attributed to the ability of PAHs to interact with π electrons associated with the aromatic portion of HSs (Gauthier et al., 1987). Thus, a similar binding mechanism is considered to be involved in the association of BaP to HA. Other than aromatic properties, it has also been suggested that the atomic O/C ratio (Gauthier et al., 1987; Huang and Weber, 1997; Huang et al., 2003), aliphaticity (Chefetz,

Table 5 The correlation coefficient between K_{oc} value or median mitigating concentration (MMC) and chemical properties of HAs.

	K_{oc}		MMC	
	Six HA	**Four HA**	**Six HA**	**Four HA**
$\mathbf{K_{oc}}$	-	-	-0.751	-0.953**
MMC	-0.751	-0.953*	-	-
H/C	-0.688	-0.967**	0.440	0.998**
O/H	0.759*	0.893*	-0.450	-0.976**
DU	0.747	0.965**	-0.449	-0.998**
Ash	0.467	-0.068	0.269	0.004
Alkyl-C	-0.747	-0.974**	0.339	0.996**
Aryl-C	0.702	0.987**	-0.550	-0.902*
Aromaticity	0.639	0.994**	-0.354	-0.940*
∑Alkyl	-0.648	-0.988**	0.281	0.940*
∑Aryl	0.562	0.995**	-0.449	-0.934*
∑Aryl/∑Alkyl	0.786*	0.967**	-0.464	-0.845

* (p<0.05), **(p<0.01), ∑Alkyl: Alkyl-C + O-Alkyl-C, ∑Aryl: Aryl-C + Phenolic-C.
Four HAs are except for two Histosols HAs.

2000; Salloum et al. 2002), both atomic O/C ratio and aliphaticity (Zhang et al., 2009b), atomic H/O ratio (Grathwohl, 1990) or polarity (Rutherford et al., 1992; Kile et al. 1999) are involved in the binding affinity between PAHs and HS. No relationship with structural characteristics has previously been described in the literature, and full agreement among researchers has not yet been achieved (Ahangar, 2010). However, it is considered that a specific structure of HA relates to the binding affinity to BaP, because the HAs with a wide range of characteristics in this study also showed tendencies similar to those in previous reports.

In contrast, Histosols HAs in our study showed a tendency to deviate slightly from this relationship. In fact, when the four non-Histosols HAs were examined for a relationship between K_{oc} values and structural characteristics, correlations with many structural characteristics were obtained (Table 5). Histosols HAs differ in formation process from Andosols and Cambisols HAs. Such a difference is considered to affect the partial structure, which cannot be represented by averaged structural information such as NMR spectra and elemental analysis. Perminova et al. (1999) also reported that the structural descriptions that correlated to the binding affinity of three PAHs differ depending on the origin of HS. In addition, Golding et al. (2005) suggested that not all the aromatic moieties are completely involved in binding to PAHs. The involvement of physical structural characteristics such as molecular size, intramolecular cavities, and physical conformation have also been suggested (McCarthy et al., 1989; Döring and Marschner, 1998; Lueking et al., 2000; Pan et al., 2008; Kobayashi and Sumida, 2015). Therefore, it is presumed that the conformational diversity and the state of the functional Groups are also associated with the affinity of HS to BaP.

Detoxifying effect of HAs on BaP phototoxicity

The mitigating effects of HS and dissolved organic matter (DOM) on bioavailability and toxicity of xenobiotics have frequently been demonstrated. Our findings are consistent with these results, as mitigating effects of BaP toxicity to *P. subcapitata* were observed in the presence of all the HAs used in this study. Perminova et al. (2001) found that the toxicities of three tested PAHs (pyrene, anthracene, and fluoranthene) are correlated with the binding affinity to HS and that the aromaticity of HS can explain most of the variability in reduction of PAH toxicity. They suggested that the interaction of HS to PAHs allows the formation of new associations and increases the molecular size. The associations are less bioavailable and thus, less bioaccumulative. The reduced bioaccumulation leads to reduced exposure of the target organism to potentially harmful substances, and consequently may result in lower harmful effects (Steinberg, 2003). Lou et al. (2006) examined the photodegradation effects of binding BaP to river and soil HS and river water DOM, and found that all organic matter samples showed a decrease in K_{oc} values after irradiation, but to a sample-specific degree. Since the sorption experiment in the present study was conducted under dark conditions, binding properties may have changed during the phototoxicity test. There were no clear relationships between the structural properties of the six HAs and the MMC values (Table 5). However, Andosols HAs with high aromaticity showed high detoxification effects, while Cambisols HAs, showing relatively low detoxification effects, had low aromaticity. In addition, the detoxification effects of Histosols HAs (low K_{oc} values but higher aromaticity) were low. Although the aromaticity level cannot be explained, we therefore suggest that the binding between HA and BaP is to some extent involved in the mechanism of reducing BaP toxicity to *P. subcapitata*, which allows a certain degree of prediction.

The mitigating toxicity effect for Cambisols and Andosols HAs increased with increased HA concentration, whereas the growth inhibition of *P. subcapitata* in the presence of Histosols HAs was restored at a higher HA concentration range (Figure 3). Meems et al. (2004) showed that the toxicity of the insecticide, cypermethrin, was highly reduced in the presence of low concentrations of natural organic matter and synthetic HS, and that the reduction effect did not increase with organic matter concentration. This phenomenon was ascribed to the direct effect of HS on organisms. In the present study, a control without BaP was used at each HA concentration to exclude the direct influence of HA from the results. Alga growth varied with the addition of HA, but there was no relationship to the increase in HA concentration (Figure 2). Two Histosols HAs in particular showed different effects on growth. Therefore, the direct effects of HA alone cannot explain the results in the present study. Growth inhibition of *P. subcapitata* by BaP is enhanced by the intense toxicity of quinones and diols produced by the alga as photooxidation products or metabolites (Cody et al., 1984; Schoeny et al., 1988). HS as well BaP is also subject to photochemical alteration, photodegrading into products that are more labile and of lower molecular weight (Moran and Zepp, 1997; Lou and Xie, 2006) and forming reactive oxygen species (e.g., singlet oxygen, peroxy radicals) (Frimmel, 1994; Aguer et al., 1999). The photodegradation of xenobiotics and HS itself is presumed to participate in toxicity development from hydrophobic organic pollutants, including PAHs in the presence of HS and light irradiation (Steinberg et al., 1992; Steinberg, 2003). Furthermore, it has also been established that photooxidation can alter both PAH and HS to produce daughter products (Steinberg et al., 1992; Wang et al., 1995; Gensemer et al., 1998). The resulting decrease or increase in toxicity is presumed to be governed by the interaction of the original PAH and HS, each daughter product, and the daughter products formed from PAH bound to HS.

Histosols HAs showed the highest phenolic moiety contents (Table 4). The lignin constituents in HAs are most affected by photooxidation (Schmitt-Kopplin et al., 1998). Paul et al. (2004) demonstrated that Histosols HAs showed higher quantum yields from singlet oxygen photogeneration compared to mineral soil HAs. We suggest that due to higher photosensitivity, the Histosols HAs in the present study generated the highly toxic substance from the interaction with BaP and showed a tendency for toxicity changes different from the mineral soil-derived Cambisols and Andosols HAs.

Conclusion

The results of the present study suggest that the binding affinity of BaP to three selected HAs was related to the aromatic moiety of the HAs. However, Histosols HAs tended to deviate slightly from this relationship. In addition, the phototoxicity of BaP to the green alga *P. subcapitata* was mitigated in the presence of any tested HA. Although no clear relationships between the structural properties of the six HAs and the mitigating effect were detected, it was inferred that the binding of HA and BaP is to a certain extent involved in the mechanism of reducing BaP toxicity to *P. subcapitata*. On the other hand, the restoration of growth inhibition at a higher HA concentration range occurred only in the presence of Histosols HAs. We conclude that the difference in tendency for binding affinity and toxicity development among the HAs, which were derived from three different soil types, was influenced by their diversity of steric structure, the state of the functional Group, and the photosensitivity of HAs depending on the formation process. We therefore suggest that to evaluate the influence of HAs on the phototoxic behaviour of BaP, it is necessary to consider differences in formation process and partial structure.

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