

Pyrene and nile red fluorescence probes for *in-situ* study of polarity and viscosity of soil organic coatings implicated in soil water repellency

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Abstract

Soil water repellency, that is, the reduced ability of soils to absorb water, is thought to be caused by organic coatings with predominantly non-polar properties on soil particle surfaces. Given the important role of particle surface polarity in determining soil water repellency, we explored the use of fluorescent probes as a method for the direct *in-situ* determination of the distribution and polarity of organics on bulk soil surfaces, and of their molecular mobility. We used nile red and pyrene, which have both been used successfully as environmental probes in previous studies, but have not been applied before to bulk soils. The probes were either (a) co-deposited with other organics known to induce water-repellent behaviour with acid-washed sand to produce 'model soils' or (b) adsorbed directly onto sandy soils that were naturally water repellent to different degrees, and studied using fluorescence microscopy and steady-state and time-resolved fluorescence. Reliable measurements could be made using pyrene as an *in-situ* probe on both model and natural soils, and a viscosity/mobility probe on model soils, whereas nile red was found not to be a useful probe. On model soils, made using hexadecane (HEX), octadecane (OCT) or stearic acid (SA) on acid-washed sand, pyrene excimer formation kinetics showed a decrease in environment mobility as the organic layer changes from a liquid through to a hard wax. Spectra from pyrene adsorbed to natural soils indicated varying environmental polarity and heterogeneity within the soil samples studied.

Highlights

- Exploration of *in-situ* fluorescent probes to study polarity and viscosity of organics on soils.
- Fluorescent probes have never been used *in situ* on bulk soils before.
- Pyrene probe shows variation in mobility, polarity and heterogeneity of organics on soils.
- Pyrene is a useful *in-situ* fluorescent probe of polarity and mobility of organics on soils.

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KEYWORDS

autofluorescence, emission lifetime, emission spectrum, excimer, hydrophobicity, water repellent

1 | INTRODUCTION

The nature of the substances adsorbed onto a soil surface is relevant to many soil properties, such as nutrient/contaminant fixation and mobility, as well as our particular area of interest: water repellency. Soil water repellency is the reduced ability of affected soils to absorb water and become wetted. It can have substantial environmental consequences, such as increased overland flow leading to flooding, soil erosion and poor uptake of agricultural chemicals (Doerr, Shakesby, & Walsh, 2000). The latter increases the risk of crop disease, reduces yields and threatens food security and production (Bond, 1972). Soil water repellency also increases the risk of groundwater pollution by accelerating contaminant and nutrient leaching (Bisdorn, Dekker, & Schoute, 1993; Ritsema & Dekker, 1996; Hallett, Douglas, Ritz, Wheatley, & Young, 2001) via uneven wetting and preferential flow pathways (Dekker & Ritsema, 1994; Dekker, Ritsema, & Oostindie, 2000). It is thought to be caused by organic compounds with hydrophobic (non-polar) properties present as coatings on soil grains (Bisdorn et al., 1993; Doerr et al., 2000; Roberts & Carbon, 1972) and in interstitial matter (Franco, Clarke, Tate, & Oades, 2000). These compounds can be derived from leaf surface waxes (McIntosh & Horne, 1994), fungal and microbial activity (Jex, Bleakley, Hubbell, & Munro, 1985; Hallett et al., 2001), plant roots (Dekker & Ritsema, 1996; Doerr, Shakesby, & Walsh, 1998) and lipids from decomposing litter (McGhie & Posner, 1981). For sandy soils, the main cause of soil water repellency is believed to be these compounds adsorbed at soil particle surfaces (Bisdorn et al., 1993; Doerr et al., 2000; Mainwaring et al., 2004; Mainwaring, Hallin, Douglas, Doerr, & Morley, 2013). However, a direct relationship between the degree of soil water repellency and either quantity or type of organic compound (non-polar or amphiphilic) has not been established, although there is evidence that particular combinations of organic compounds are more effective at inducing water repellency than individual compounds (de Blas, Almendros, & Sanz, 2013; Mainwaring et al., 2013; Mao, Nierop, Rietkerk, Sinninghe Damaste, & Dekker, 2016). Even so, in a general sense, the polarity of the organics adsorbed to a soil might be expected to influence water repellency, with a polar, hydrophilic, layer giving a wettable soil, and a very non-polar, hydrophobic, layer giving a highly repellent soil. It might be expected that organic polarity would influence the initial soil–water interaction/contact angle and, because

wetting is often not an instantaneous process, both polarity and molecular mobility might be expected to influence water drop penetration time (WDPT). Thus, the polarity and molecular mobility (viscosity) of this organic layer may well be important in influencing soil water repellency.

Fluorescent probes have been successfully used for decades to study biological and chemical environments (Evans, Douglas, & Burrow, 2013). We explore the use of fluorescent probes as a method for the direct *in-situ* determination of the distribution and polarity of organics on soil surfaces and their molecular mobility. Here, we focus on nile red and pyrene. Nile red is an environment polarity probe, which shows an increase in both absorption and emission wavelength maxima with increasing polarity, from non-polar (e.g., hexane (484 nm_{abs}, 526 nm_{em})) to polar (e.g., methanol (549 nm_{abs}, 633 nm_{em})) environments, and a marked decrease in fluorescence emission intensity in very polar environments such as water (Deye, Berger, & Anderson, 1990; Zhang & Cai, 2011). Pyrene is both a polarity and viscosity probe. An outline of the basic photochemistry of pyrene is given in the Supporting information. For this work, the important features are: (a) pyrene monomer fluorescence shows a series of vibrational bands, the relative intensities of which vary with environment polarity (Kalayanasundaram & Thomas, 1977); and (b) the kinetics of pyrene excimer formation can be used to assess environment viscosity (Costa, Gomes, Pillinger, Goncalves, & Seixas de Melo, 2015; Glushko, Thaler, & Karp, 1981). Pyrene excimer formation has been widely studied in many different environments (Birks, Dyson, & Munro, 1963; Birks, Lumb, & Munro, 1964; Montalti, Credi, Prodi, & Gandolfi, 2006). In fluid solution, excimer formation occurs at every molecular encounter between an excited-state and ground-state pyrene molecule. If excimer formation involves diffusion of the reacting species to come together, this is described as dynamic excimer formation, but if it happens because the two pyrene molecules are already sitting next to one another it is described as static excimer formation (Evans et al., 2013). In emission lifetime studies, dynamic excimer formation is shown by an increase in excimer concentration and, therefore, emission intensity at the excimer emission wavelength, in the initial time period after the excitation pulse; for static excimer formation, the excimer is formed within the excitation pulse and, therefore, excimer emission intensity decays monotonically after excitation.

Pyrene has previously been used as a polarity probe on extracted soil organics (Ganaye, Keiding, Viriot, & Block, 1997). In a previous work in our laboratory, confocal laser microscopy was used to examine single soil grains after adsorption of either Nile red or Nile blue (Bayer, 2009), but because of signal saturation little could be concluded from this work. We are not aware of any studies of probes used directly on bulk soils. The work presented here focused on the following two questions. (a) Can reliable measurements be made on pyrene and Nile red as *in-situ* fluorescent probes? (b) If so, what information can such measurements offer on the nature and properties of the probed environment and, by extension, the nature and properties of organics present on the soil grains?

With Nile red we wanted to explore whether this probe could be used to: (a) identify localized areas of organics by visualization of localized adsorption of the probe, and (b) obtain some idea of the general polarity of the Nile red environment, and hence, by extension, some idea of the polarity of the organics at the soil surface.

Pyrene was used to examine whether it could provide: (a) a measure of the viscosity of the organic layer surrounding soil grains and model soils, and (b) a measure of the polarity of the pyrene environment, and hence, by extension, the polarity of the organics at the soil surface. The probes were either (a) co-deposited with organics for 'model soil' studies or (b) adsorbed directly onto natural soils, and studied using fluorescence microscopy and steady-state and fluorescence lifetime studies. The adsorption of pyrene onto natural soils (and acid-washed sand, to provide a 'blank' with no organics) was carried out to allow pyrene infiltration into the organic layer, with the expectation that it would be taken in by the organics present. The addition of pyrene via co-deposition with organics onto acid-washed sand was used to assess the use of pyrene as a probe for the mobility of the organic layer at the soil surface. The organics used were: stearic acid (SA) (C_{18} amphiphilic, m.pt 69°C), hexadecane (HEX) (C_{16} non-polar linear hydrocarbon, m.pt 18°C), octadecane (OCT) (C_{18} non-polar linear hydrocarbon, m.pt 28°C) and squalane (SQ) (C_{30} non-polar branched hydrocarbon, m.pt -38°C), which might be expected to give a range of properties ranging from a non-polar liquid (HEX, SQ) to a non-polar soft wax (OCT) to a harder, amphiphilic, wax (SA).

2 | METHODS AND MATERIALS

2.1 | Materials

Nile red (Sigma Aldrich, Dorset, UK, Bioreagent, 98%), pyrene (Fluka Chemika, Buch, Switzerland, 99%), SA (British Drug Houses, Poole, UK, 98%), OCT (British Drug

Houses, Poole, UK, general purpose, 99%), HEX (British Drug Houses, Poole, UK, 99%), SQ (British Drug Houses, Poole, UK), ethanol (Fisher Scientific, Loughborough, UK, analytical reagent), acetone (Fisher Scientific, Loughborough, UK, laboratory grade) and acid-washed sand (Fisher Scientific, Loughborough, UK) were all used as received. Distilled water was used throughout. Samples of sandy soils (0–10 cm depth) from six naturally water-repellent soils were used.

2.2 | Soil sampling and preparation

Samples of sandy soils (0–10 cm depth) were taken from six naturally water-repellent soils (Balshaw, 2019; Doerr et al., 2005). Four were from Gower, South Wales: two dune soils from Nicholaston, NIC1 and NIC2 ($51^{\circ}34'N$ $04^{\circ}07'W$), and two dune soils under pine forest from Llanmadoc, LLAN1 and LLAN2 ($51^{\circ}37'N$ $04^{\circ}15'W$). Two soils from Australia, AU2 ($36^{\circ}26'S$ $140^{\circ}40'E$) and AU3 ($36^{\circ}26'S$ $140^{\circ}41'E$), were also included as they had been examined in previous work by our group (Doerr et al., 2005). Details are given in Table 1. After collection, soils were oven dried at 30°C for 48 hr and then sieved using a 2-mm sieve to remove any large pieces of organic debris.

2.2.1 | Preparation of basic and acidic soil surfaces

Given that our blank reference material was acid-washed sand it was of interest to examine the effect of surface acidity on probe response. For visualization experiments with Nile red, AU2 and AU3 soils were stripped of their organics using procedures given by Mainwaring (2004), to determine whether there was any differentiation between Nile red emission from an acidic surface compared to a basic surface. To strip organics and leave a basic soil surface, 100 mL of 1 M NaOH solution was added to 50 g of 2-mm sieved, oven-dried (30°C) soil and stirred for 30 min. The solution was decanted, 400 mL of water added, and the sample left overnight. Rinses of 100 mL of distilled water were used until a rinse water pH of ~ 7.0 – 8.0 was achieved; the soil was collected by filtration under vacuum and then oven dried at 100°C for 24 hr. To create an acidic soil surface, 100 mL of 0.1 M HCl was added to approximately 25 g of NaOH-stripped soil and stirred for 5–10 min. The sample was rinsed three times with distilled water until the pH of the rinse water reached ~ 7.0 ; the soil was collected by filtration under vacuum and then oven dried at 100°C for 24 hr.

TABLE 1 Source locations and characteristics of the naturally water-repellent soils used

Code	Country	Site location, region	Lat/long	Vegetation type	Sample depth (cm)	Mean diameter (mm)	^a Total carbon content/ g kg ⁻¹	Water repellency class ^d
AU2	Australia	Pine Views, Naracoorte	36°26'S 140°40'E	Cropland	0–10	0.29	^a 5.1 (±1.1)	Strongly
AU3	Australia	Pine Views, Naracoorte	36°26'S 140°41'E	Cropland	0–10	0.23	^a 2.5 (±0.5)	Extremely
NIC1	Wales	Nicholaston, Gower	51°34'N 4°7'W	Dune grass	0–10	0.32	^b 4.5 (±0.3)	Severely
NIC2	Wales	Nicholaston, Gower	51°34'N 4°7'W	Dune grass	0–10	0.33	^b 6.7 (±0.7)	Strongly
LLAN1	Wales	Llanmadoc, Gower	51°37'N 4°15'W	Pine forest	0–10	0.27	^c 21.9 (±4.4)	Strongly
LLAN2	Wales	Llanmadoc, Gower	51°37'N 4°15'W	Pine forest	0–10	0.29	^c 19.7 (±5.3)	Strongly

^aPrevious work with AU2 and AU3 has shown the inorganic carbon content to be negligible and total carbon content to be organic in origin (Doerr et al., 2005).

^bPrevious work using soils obtained from a similar Nicholaston location recorded 27% inorganic carbon as part of the total carbon present (I. L. Hallin, personal communication, December 2, 2019). In this work the shape of the peak detection curves for total carbon analysis indicates an inorganic carbon contribution of $\leq 50\%$.

^cIn this work an assessment of the detection curves for Llanmadoc soils indicates an inorganic carbon contribution of $\leq 20\%$.

^dDetermined from water drop penetration time (WDPT) test and classification of Bisdom et al. (1993).

2.2.2 | Addition of Nile red to soils for fluorescence microscopy imaging of organics adsorbed to soils

The adsorption method was based on that of Greenspan, Mayer, and Fowler (1985) for addition of Nile red (nr) to cells. One gram of soil was added to 5 mL of distilled water and 10, 20, 50 or 100 μL of either 2.17×10^{-4} M Nile red in ethanol or 2.17×10^{-3} M Nile red in acetone were added. The sample was inverted back and forth for ca. 1 min, the water was decanted, and the sample rinsed with 1 mL of water and then placed onto a filter paper in a Petri dish and allowed to dry. The highest amount used gave a laydown of 2.17×10^{-7} mol_{nr} g⁻¹_{sand}; 6.91×10^{-5} g_{nr} g⁻¹_{sand}, assuming complete adsorption of Nile red. *Notes:* When quantifying laydowns subscript 'sand' is used to indicate either 'acid-washed sand', or 'sandy soil', as appropriate. Throughout the text, laydowns are given assuming complete deposition of all the organic and probe added. Although this is a good approximation for co-deposition with rotary evaporation to remove solvent (Hallin et al., 2017; Mainwaring et al., 2013), it might not be the case for adsorption onto soils from aqueous solution.

2.2.3 | Addition of pyrene to soils

Co-deposition of pyrene to acid-washed sand

The addition of pyrene via co-deposition with organics onto acid-washed sand was used to assess the use of

pyrene as a probe for the mobility of the organic layer at the soil surface. For the model soils we took the method previously used to deposit organics onto acid-washed sand (Ma'Shum, Tate, Jones, & Oades, 1988; Mainwaring et al., 2013). The organic compounds used were: SQ, which is a branched highly non-polar low melting point (m.pt) (-38°C) liquid, not found in natural soils but used to provide an unambiguous non-polar liquid phase when adsorbed onto soil grains; HEX and OCT, which are two linear highly non-polar hydrocarbons of slightly different chain lengths, with m.pts around ambient temperatures (18°C HEX and 28°C OCT) and found in natural soils (Doerr et al., 2005); and SA (m.pt 69°C), which is an amphiphilic molecule with a long non-polar hydrocarbon chain attached to a polar carboxylic acid 'head group' also found in natural soils (Doerr et al., 2005).

Five grams of acid-washed sand, or natural soil, and 10 mL ethanol were placed in a round bottom flask, followed by 10 mL of ca. 10^{-3} M of one of the organics in ethanol (1.03×10^{-3} M SA, 9.7×10^{-4} M OCT, 9.1×10^{-4} M HEX and 1.03×10^{-3} M SQ) and 50 μL of 1.01×10^{-3} M pyrene in ethanol. Solvent was removed by rotary evaporation (40°C , 120 rpm increasing to 240 rpm, to dryness plus 15 min) until the sand/soil was dry and flowing freely within the flask. Assuming complete deposition of organics, this gave molar laydowns per gram of sand/soil of $\sim 2 \times 10^{-6}$ mol_{organic} g⁻¹_{sand} (2.06×10^{-6} mol_{SA} g⁻¹_{sand}, 1.94×10^{-6} mol_{OCT} g⁻¹_{sand}, 1.82×10^{-6} mol_{HEX} g⁻¹_{sand} and 2.06×10^{-6} mol_{SQ} g⁻¹_{sand}), and mass laydowns of 5.86×10^{-4} g_{SA} g⁻¹_{sand}, 4.94×10^{-4}

$g_{\text{OCT}} g_{\text{sand}}^{-1}$, $4.12 \times 10^{-4} g_{\text{HEX}} g_{\text{sand}}^{-1}$ or $8.71 \times 10^{-4} g_{\text{SQ}} g_{\text{sand}}^{-1}$. The ratio of pyrene to organics gave pyrene concentrations of ca. 0.009–0.019 M (0.013 M_{SA} , 0.016 M_{OCT} , 0.019 M_{HEX} , 0.009 M_{SQ}), assuming a bulk solution. The specific surface area of acid-washed sand used was $292 \pm 3 \text{ cm}^2 g^{-1}$ (Hallin et al., 2017), which, by way of example, would give a ~21-nm-thick layer of SA on acid-washed sand at the highest laydown of SA used.

Adsorption of pyrene on to soils

The adsorption of pyrene onto natural soils (and acid-washed sand to provide a 'blank' with no organics) was carried out to allow pyrene infiltration, with the expectation that it would be taken in by the organics present. Five grams of soil was mixed with 25 mL distilled water and allowed to settle for 1 min. Five hundred microlitres of $1.01 \times 10^{-3} \text{ M}$ pyrene (py) in ethanol was added. This amount was chosen as a compromise between the lowest possible probe concentration and adequate signal to background and noise ratio. This gave a molar laydown of $1.01 \times 10^{-7} \text{ mol}_{\text{py}} g_{\text{sand}}^{-1}$ and a mass laydown of $2.04 \times 10^{-5} g_{\text{py}} g_{\text{sand}}^{-1}$. The sample was covered with parafilm® to prevent evaporation, stirred for 3 hr, filtered using a vacuum pump, washed twice with water, dried further on the vacuum pump for 15 min, placed on a filter paper in a Petri dish and allowed to dry overnight.

2.2.4 | Fluorescence microscopy for imaging of Nile red adsorbed to soils and soil organics

Fluorescence microscopy with vertical (episcopic) illumination was carried out using an Olympus BX51 microscope with a medium pressure mercury arc lamp and Olympus Soft Imaging Solutions XC10 camera. All images were recorded with an exposure time of 115.5 ms and camera sensitivity (gain) of '0'. Any subsequent image manipulation is described in the text. A $\times 4$ Olympus objective was used for most imaging, with $\times 20$ for spectral analysis of soil auto-luminescence. Three illumination/detection filter (Thorlab) arrangements were used.

Green light excitation, red light emission (green_{ex}/red_{em})

Excitation filter maximum 559 nm, 34 nm bandwidth; a dichroic reflecting 533–580 nm and transmitting 595–800 nm; with a 665 nm longpass emission filter.

Near UV excitation, visible wavelength emission (UV_{ex}/vis_{em})

Excitation filter maximum 390 nm, 18 nm bandwidth; a dichroic 360–407 nm, with a 435 nm longpass emission filter to allow imaging across the visible spectrum.

Room light excitation, visible wavelength emission

(roomlight_{ex}/vis_{em})

For room light (fluorescent tube light) excitation no emission filters were used to allow imaging across the visible spectrum.

Two other excitation filters, 475 nm with 35-nm bandwidth and 497 nm with 16-nm bandwidth, which would be expected to give images from Nile red in very non-polar environments, were also tried but gave no useful images.

2.2.5 | Soil auto-luminescence spectroscopy for 'background' emission

Spectral analysis of soil auto-luminescence was carried out through the BX51 microscope by collecting the light from the microscope eyepiece using a 1-mm-diameter fibre optic connected to a HR2000+ Ocean Optics spectrometer.

2.2.6 | Transmission microscopy of organics on soil grains

An Olympus BH3 microscope was used for transmission microscopy of organics on natural soil grains. Analysis through crossed polarisers gave some advantage because quartz grains are bright through crossed polarisers whereas organics are dark.

2.2.7 | Fluorescence spectroscopy of pyrene and Nile red

A Horiba FluoroMax-4 fluorimeter with front face accessory was used with samples held in 1 or 2-mm path length quartz cuvettes. For pyrene studies, 15 nm excitation slits and 0.5 nm emission slits were used, with a 337 nm centre wavelength (CWL), 10 nm full-width half maximum (FWHM), bandpass interference filter (Edmund Optics) to reduce scattered excitation light (without this filter, spectra showed too high a background signal to be useful). For Nile red, a 532-nm filter was used, although this was much less critical. Spectra were corrected for detector response using files provided by the manufacturer. For this particular instrument, front face and 90° detection gave slightly different spectra for the same sample. The reason for this is not clear, but where spectra have been corrected for this, using data from the same samples recorded using both front face and 90° detection, this is stated in the text. For most experiments two to three independent runs were obtained and the data summed.

2.2.8 | Fluorescence lifetime measurements of pyrene

Lifetime data were collected at the Chemistry Department, University of Coimbra. Fluorescence decays were measured using a home-built time-correlated single photon counting (TCSPC) apparatus. The excitation source was a Horiba-Jobin-Yvon nanoled with $\lambda_{\text{exc}} = 337$ nm with the 337 nm CWL (10 nm FWHM) bandpass interference filter used for fluorimetry above. Emission at 90° geometry was collected through a double subtractive Oriol Cornerstone 260 monochromator and detected by a Hamamatsu microchannel plate photomultiplier (R3809U-50). A longpass glass colour filter with transmittance onset at 375 nm was used in the emission optical path to discard scattered light from the sample. Signal acquisition and data processing were performed using a Becker & Hickl SPC-630 TCSPC module. The fluorescence decays and the instrumental response function were collected using a timescale of 1,024 channels with 400 ps/channel. The samples were slightly pressed in a circular (10 mm) powdered sample holder well (without quartz window) positioned at a 45° angle with respect to

the TCSPC emission path to avoid interference from the excitation source. Experiments were carried out at 19–20°C. Deconvolution of the fluorescence curves was performed using the modulating function method as implemented by Striker, Subramaniam, Siedel, and Volkmer (1999) in the SAND program.

3 | RESULTS AND DISCUSSION

3.1 | Imaging of natural organics on soil grains

Transmission microscopy images, with parallel and crossed polarisers, of naturally occurring organics on unmodified soil grains (AU2) are given in the Supporting information. Localized organics can be clearly seen as dark spots, along with what appears to be bare mineral surfaces. Based on previous studies (Horne & McIntosh, 2000; Atanassova & Doerr, 2010; de Blas et al., 2013), these apparently bare mineral surfaces may also be covered in an optically undetectable coating of organics.

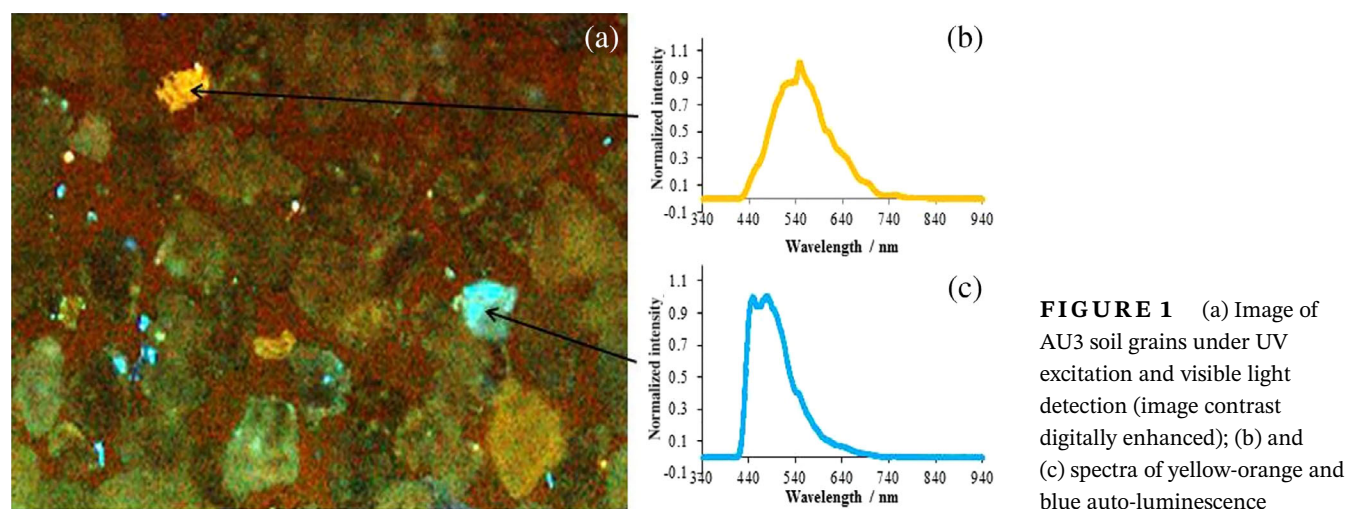


FIGURE 1 (a) Image of AU3 soil grains under UV excitation and visible light detection (image contrast digitally enhanced); (b) and (c) spectra of yellow-orange and blue auto-luminescence

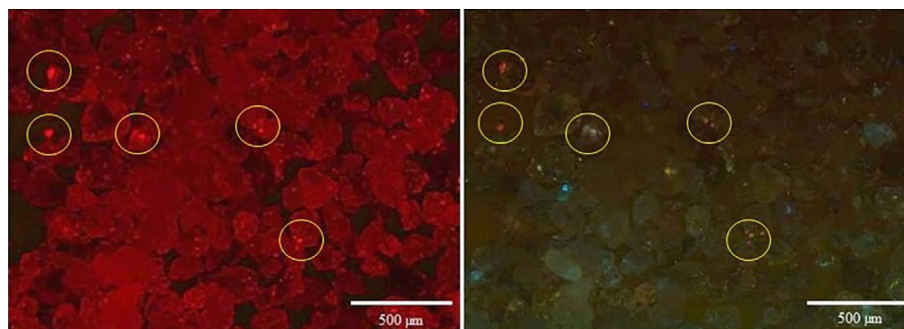
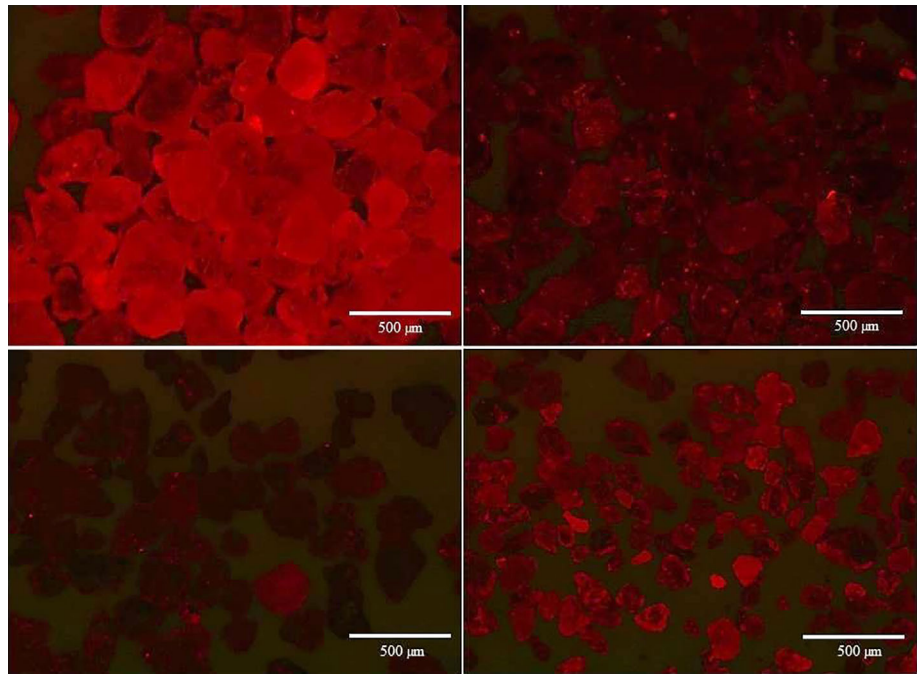


FIGURE 2 Fluorescence microscopy images of AU3 soil grains treated with Nile red ($6.91 \times 10^{-5} \text{ g}_{\text{nr}} \text{ g}_{\text{sand}}^{-1}$), under green (left) and UV light (right) excitation. Image contrast has had saturation, gamma and brightness digitally enhanced. Examples of regions of high-intensity emission under both green and UV light are highlighted to illustrate the effect of auto-luminescence

FIGURE 3 Fluorescence microscopy images of: acid-washed sand (top left) and AU3 soil grains (top right) under green excitation light, both prepared by adsorption using $3.45 \times 10^{-6} \text{ g}_{\text{nr}} \text{ g}^{-1} \text{ sand}$, and base stripped/basic surface (bottom left) and base stripped/acidic surface (bottom right) AU3 soil grains with same application of Nile red. Image saturation, gamma and brightness have been digitally enhanced simultaneously for all images



3.2 | Soil auto-luminescence

A typical emission image from a natural soil (AU3) with UV excitation is shown in Figure 1a. Throughout the soil, individual grains, and sometimes parts of grains, gave intense emission, with colours varying from blue to red. Emission spectra for two of these grains are given in Figure 1b and c. The emission spectra are very broad and, significantly for this work, extend across the visible spectrum into the red. (The apparent structure in the spectra is due to atomic emission lines from the mercury lamp). Images from both $\text{UV}_{\text{ex}}/\text{vis}_{\text{em}}$ and $\text{green}_{\text{ex}}/\text{red}_{\text{em}}$ of the same collection of AU3 soil grains with Nile red deposited are shown in Figure 2. Unfortunately, even with the microscope filters set for $\text{green}_{\text{ex}}/\text{red}_{\text{em}}$, soil auto-luminescence generated regions of bright red emission (in the same spectral region expected for the Nile red fluorescence probe), thus creating the possibility of interference when using Nile red, by a strong false-positive Nile red probe response in regions of high auto-luminescence.

3.3 | Nile red probe imaging

Fluorescence microscopy images of untreated AU3, acidic and basic AU3 soil after base stripping of organics, and acid-washed sand, all with Nile red at the same laydown, under $\text{green}_{\text{ex}}/\text{red}_{\text{em}}$ are shown in Figure 3. Nile red adsorbed onto acidic surfaces gave relatively high emission intensity, whereas the basic soil surface

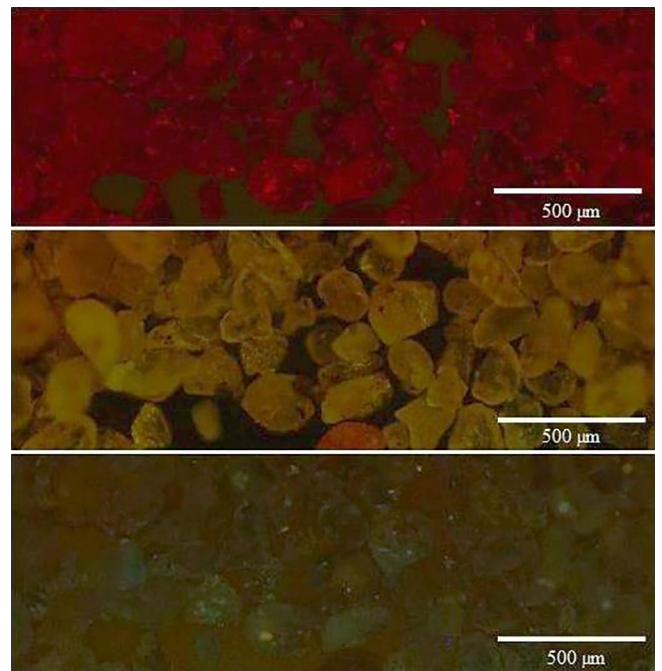


FIGURE 4 Fluorescence microscopy images of AU2 soil grains prepared by adsorption using $3.45 \times 10^{-6} \text{ g}_{\text{nr}} \text{ g}^{-1} \text{ sand}$ Nile red under $\text{green}_{\text{ex}}/\text{red}_{\text{em}}$ (top), $\text{room light}_{\text{ex}}/\text{vis}_{\text{em}}$ (middle) and $\text{UV}_{\text{ex}}/\text{vis}_{\text{em}}$ (bottom). Image saturation, gamma and brightness have been digitally enhanced

either did not adsorb Nile red significantly, or, if it did, the probe was in a relatively non-fluorescent state. Natural soils sit somewhere between the two in terms of emission intensity. Images of AU2 with Nile red adsorbed

under green_{ex}/red_{em}, room light_{ex}/vis_{em} and UV_{ex}/vis_{em} are given in Figure 4. Localized adsorption of Nile red could be seen when viewed under room light but these regions did not give high red emission intensity.

In terms of elucidating the distribution and polarity of organics adsorbed to soils, fluorescence imaging with Nile red was not promising for the soils studied here. The three main reasons were: (a) interference by soil auto-luminescence gave regions of intense false-positive signals for probe adsorption; (b) acidic sand surfaces adsorb Nile red to give a relatively highly fluorescence emission even in the absence of any organics; and (c) although Nile red was adsorbed strongly in localized sites, as shown by room light microscopy (see Figure 4, middle panel), these areas were not highly fluorescent under the filter arrangements used here.

3.4 | Pyrene probe studies

3.4.1 | Model soils

Fluorescence spectra of pyrene, when co-deposited with SQ, SA, HEX or OCT, are shown in Figure 5. All spectra show the fine structure of monomer emission around 360–420 nm and broad excimer emission across ~420–600 nm. Although total emission intensities are comparable, differing only by a factor ~2, the relative intensities of monomer to excimer emission differ substantially. The ratio III/I of pyrene emission bands is used as a measure of polarity (Kalayanasundaram & Thomas, 1977). The ratio III/I generally increased in order of decreasing polarity: SA (amphiphilic) (III/I = 1.33) < OCT (linear C₁₈ hydrocarbon) (III/I = 1.47) < HEX (linear C₁₆ hydrocarbon) (III/I = 1.71) < SQ (branched C₃₀ hydrocarbon) (III/I = 1.89). We note that the original work of Kalayanasundaram and Thomas (1977) gives comparable data, with I₃/I₁ = 1.67 for pyrene in dodecane solvent (linear C₁₂ hydrocarbon) and III/I = 1.74 for pyrene in squalene solvent (branched C₃₀ hydrocarbon); the reason for the lower ratio for OCT compared to HEX, found here, is not yet known.

In terms of our two initial questions, these studies of pyrene in organics of known polarity adsorbed on to acid-washed sand give a strong indication that pyrene has the potential for use as a polarity probe of organics adsorbed to model soils. Reliable measurements can be made on this type of sample and the data can be interpreted in terms of the polarity of the probe environment.

Fluorescence lifetime measurements for pyrene co-deposited with HEX, OCT and SA on acid-washed sand, measured at wavelengths corresponding to primarily monomer emission (380 nm) and primarily excimer

emission (500 nm), are presented in the Supporting information, along with a tentative kinetic analysis. Here, we need only be concerned with excimer formation and decay. Excimer formation and decay is different in all three cases: in HEX there is some initial excimer emission from static excimer formation, but then excimer emission increases significantly by dynamic formation to reach a maximum followed by monotonic decay; in OCT there is a high initial emission from static excimer formation, followed by a smaller amount of dynamic formation, after which excimer emission decays monotonically; in SA there is a high initial emission from static excimer formation, but no dynamic excimer formation, and so excimer emission decays monotonically. In HEX, a liquid at the temperature of measurement, pyrene emission showed predominantly *dynamic* formation of excimer. In SA, which is a solid at the temperature of measurement, only *static* excimer formation was observed. In OCT, which is a soft wax at the measurement temperature, both *dynamic* and *static* excimer formation were observed.

In terms of identifying molecular mobility in the organics adsorbed to soils, the data show a difference in pyrene molecular mobility within the organics adsorbed at the soil surface, consistent with a change from liquid through soft wax to rigid solid in going from HEX to OCT to SA. Overall, as the environment moved from liquid to soft wax to hard wax, there was a relative decrease in *dynamic* excimer formation. These studies of pyrene in organics of different viscosities adsorbed on to acid-washed sand give a strong indication that pyrene has the potential for use as a viscosity/mobility probe for organics adsorbed to model soils. Reliable measurements can be made on this type of sample and the data can be interpreted to give information on the viscosity of the probe environment.

Monomer emission spectra from pyrene when adsorbed onto acid-washed sand previously coated with organics (SA, HEX), and natural soils, showing vibrational bands I and III, are given in Figure 6. Acid-washed sand without any added organics (not shown) either did not adsorb pyrene or, if it did, the adsorbed pyrene was non-emissive. This shows emission detected using model soils was from pyrene either within the organic layer, or at the organic/air or organic/inorganic interfaces, but not from bare inorganic surfaces. This is a distinct advantage of using pyrene as a probe compared to Nile red, which gave the highest emission when adsorbed to acidic sites directly on the soil surface.

3.4.2 | Natural soils

The intensities of emission for pyrene adsorbed to the soil samples vary (insets of Figure 6b–d). AU2 has a

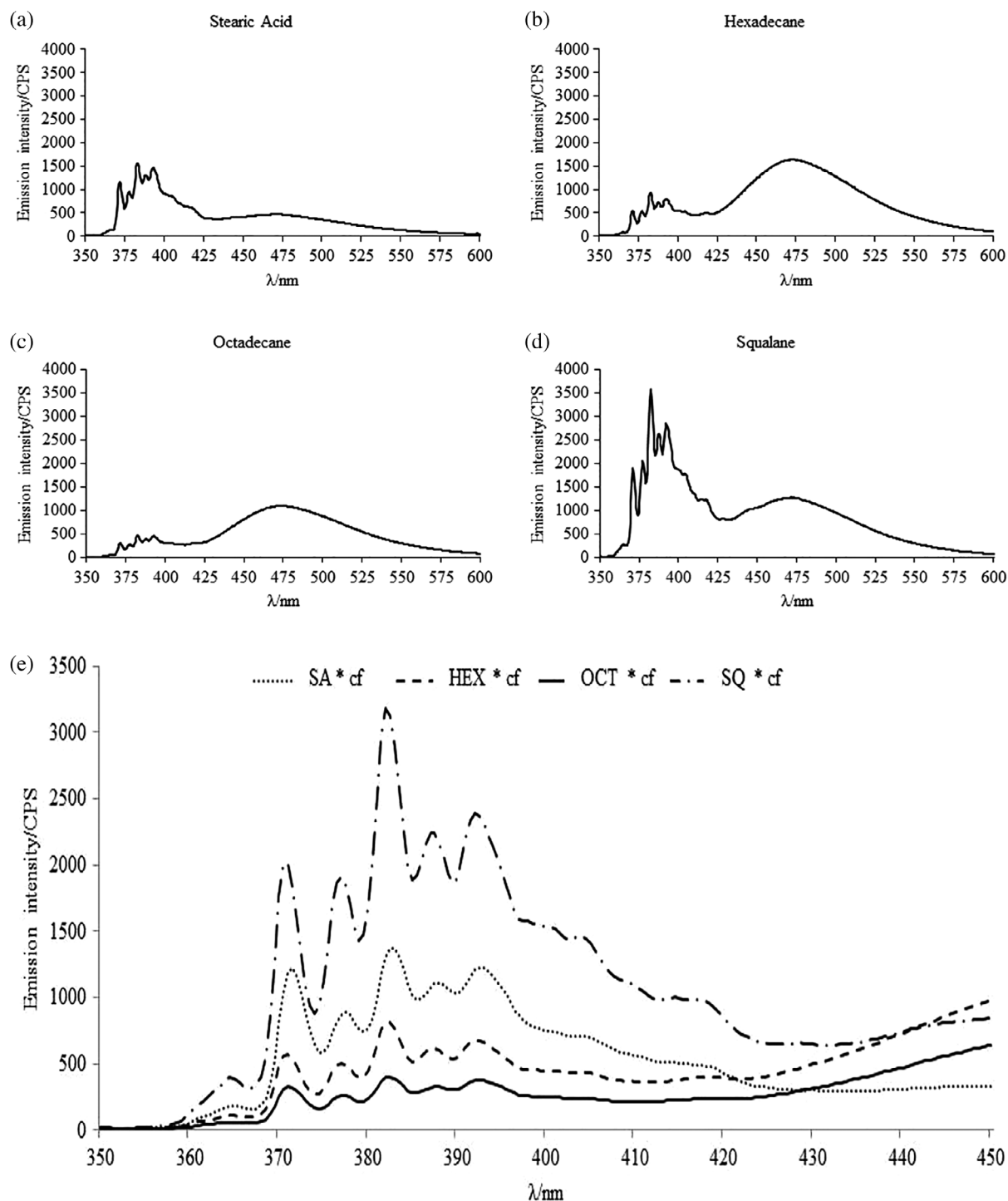


FIGURE 5 Emission spectra of pyrene co-deposited with: (a) stearic acid (SA) $5.86 \times 10^{-4} \text{ g}_{\text{SA}} \text{ g}^{-1}_{\text{sand}}$ (pyrene) = 0.013 M, (b) hexadecane (HEX) $4.12 \times 10^{-4} \text{ g}_{\text{HEX}} \text{ g}^{-1}_{\text{sand}}$ (pyrene) = 0.019 M, (c) octadecane (OCT) $4.94 \times 10^{-4} \text{ g}_{\text{OCT}} \text{ g}^{-1}_{\text{sand}}$ (pyrene) = 0.016 M, (d) squalane (SQ) $8.71 \times 10^{-4} \text{ g}_{\text{SQ}} \text{ g}^{-1}_{\text{sand}}$ (pyrene) = 0.009 M; and (e) a–d monomer emission spectra showing bands I–V, corrected for front face (*cf) from 90° measurements – emission intensities are given in counts per second (CPS)

higher organic content than AU3 but gave a lower emission intensity than AU3 (Figure 6c inset). Emission from pyrene adsorbed to natural soil was much less than that from pyrene adsorbed to acid-washed sand with SA, even though the latter had much less organic present.

Normalized spectra, shown in Figures 6b–d, allow comparison of vibration band emission intensities. In such a heterogeneous system as a soil, spectra can be expected to be weighted averages across all environments, and so some broadening of bands and loss of structure might be

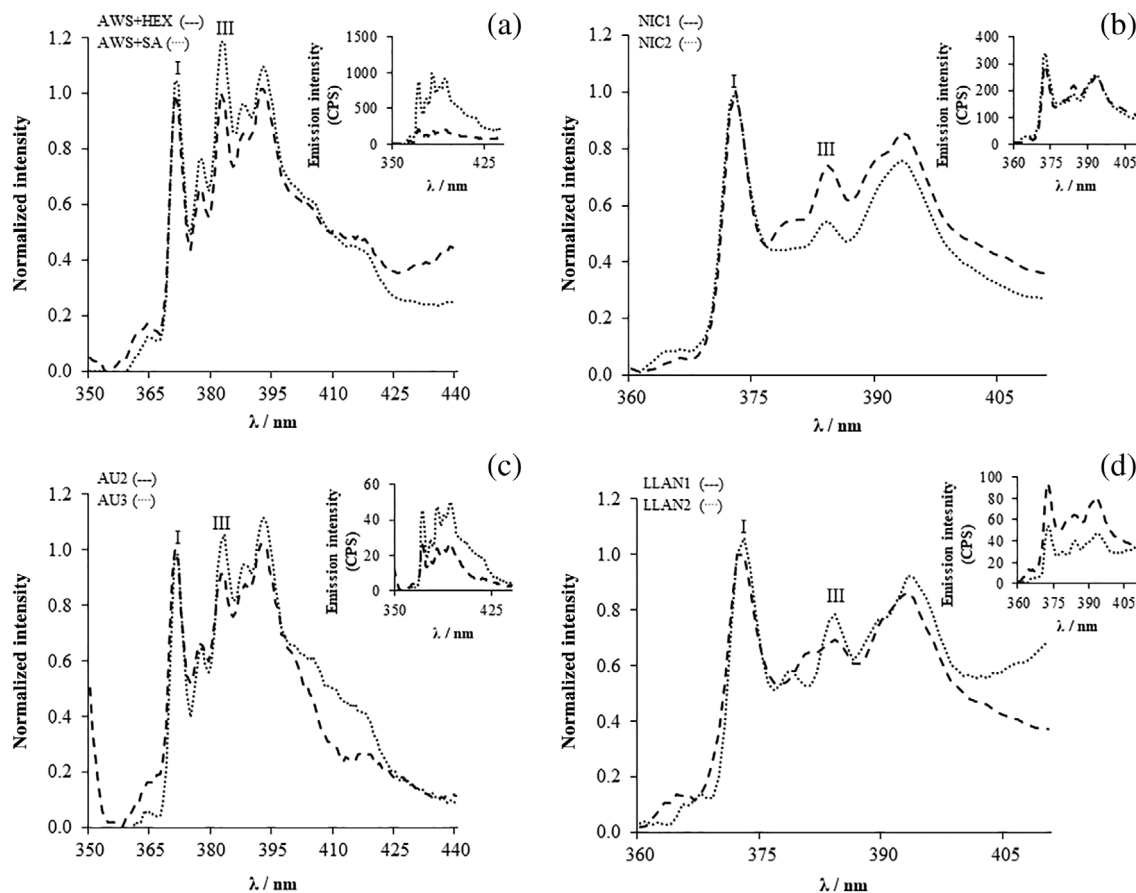


FIGURE 6 Monomer emission spectra from pyrene when adsorbed onto acid-washed sand coated with organics, and natural soils, showing vibrational bands I and III. (a) Pyrene adsorbed onto acid-washed sand (AWS) previously coated with hexadecane (HEX) ($4.12 \times 10^{-4} \text{ g}_{\text{HEX}} \text{ g}^{-1} \text{ sand}$) and stearic acid (SA) ($5.86 \times 10^{-4} \text{ g}_{\text{SA}} \text{ g}^{-1} \text{ sand}$). (b) Pyrene adsorbed onto NIC1 and NIC2, normalized summed fluorescence spectra and, insert, NIC1 and NIC2 summed emission intensity fluorescence spectra. (c) Pyrene adsorbed onto AU2 and AU3, normalized summed fluorescence spectra and, insert, AU2 and AU3 summed emission intensity fluorescence spectra. (d) Pyrene adsorbed onto LLAN1 and LLAN2, normalized summed fluorescence spectra and, insert, LLAN1 and LLAN2 summed emission intensity fluorescence spectra. Background corrected and front face corrected. Pyrene at $1.01 \times 10^{-7} \text{ mol}_{\text{py}} \text{ g}^{-1} \text{ sand}$, $2.04 \times 10^{-5} \text{ g}_{\text{py}} \text{ g}^{-1} \text{ sand}$

expected. The first thing to note is that the spectra for all soil samples differ from each other; the overall band shape, vibrational intensity ratios and structural resolution vary from sample to sample, indicating that pyrene is in different environments in these soils.

When pyrene is either co-deposited with or adsorbed on acid-washed sand with SA (laydown $1.01 \times 10^{-7} \text{ mol}_{\text{py}} \text{ g}^{-1} \text{ sand}$, $2.04 \times 10^{-5} \text{ g}_{\text{py}} \text{ g}^{-1} \text{ sand}$), the I_3/I_1 peak height band ratios are similar: all >1 , indicating a relatively non-polar probe environment (Figure 6a). With HEX the I_3/I_1 band ratio for adsorbed pyrene is significantly lower than when co-deposited, indicating a much more polar environment (Figure 6a). With the natural soils, all except AU3 show a III/I ratio <1 , indicating a relatively polar environment (AU2 0.91, NIC1 0.74, NIC2 0.54, LLAN1 0.69 and LLAN2 0.79) (Figure 6b–d). AU3 has III/I ~ 1 (1.06), indicating an environment of intermediate polarity (Figure 6c). Of all the soils, NIC2 shows a spectrum with the least obvious fine structure, perhaps

indicating a soil with a wider range of environments for pyrene adsorption (Figure 6b). As was the case for the model soils, these results suggest that pyrene has potential for use as a polarity probe for the organics adsorbed to natural soils.

4 | CONCLUSIONS

With these methods for the *in-situ* study of the polarity of soil organics, we have shown that it is possible to image emission of Nile red, and measure emission spectra and emission lifetimes for pyrene, when these probes are either adsorbed directly onto natural soils or adsorbed or co-deposited with organics onto acid-washed sand.

In terms of our initial two questions we can conclude: (a) Nile red is not a useful fluorescence imaging probe; (b) reliable measurements can be made using pyrene as an *in-situ* fluorescent probe on model and natural soils.

When using pyrene with model soils, high-quality spectra were obtained and vibration band ratios correlated well with the polarity of the organic under examination. Emission lifetime studies showed a difference in pyrene molecular mobility within the organics adsorbed at the soil surface, consistent with a change from liquid through soft wax to rigid solid in going from HEX to OCT to SA. With natural soils, adsorption of pyrene from water gave samples that also provided useable spectra. Although these were much weaker than those found for pyrene co-deposited directly with organics, and auto-luminescence gave large background signals, the spectra for the soils were all measurably different and the vibrational structure of pyrene monomer emission can be interpreted to give useful information about the polarity of the probe environment.

Further work with a wider range of soils and levels of water repellency, including perhaps different sample preparations such as prolonged drying, will be necessary to determine how well any of these spectral features correlate with water repellency and other relevant properties of natural soils. For model soils of organics on acid-washed sand, use of pyrene as a probe would appear to have immediate relevance for studies of correlations between organic composition and water repellency, as well as studies of changes in properties, as model soils are exposed to various external influences such as temperature and humidity, and as they are wetted.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study will be openly available in Cronfa at <https://doi.org/10.23889/Suthesis.52976>.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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