

Sewage Pollution: A Case Study

LABORATORY MANUAL (STUDENT)



Photos: ©C. Redshaw

Cover Photos: **Left**-Field store of lime-treated sewage biosolids, southwest England. **Right**-Adjacent field crop after biosolids treatment of soil.

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Introduction

Safe disposal of sewage is a major environmental issue for many countries. This case study seeks to illustrate how chemical markers can be used in a forensic manner to trace the fate of sewage applied initially to agricultural land, into a nearby watercourse.

Objectives

By the end of the whole exercise (sessions 1-4) you will have:

1. Been introduced to factors relating to the disposal of sewage in the U.K.
2. Identified some of the organic chemicals which can be used as markers or proxies for the presence of sewage in soil and water.
3. Learned how to safely and accurately use a variety of modern methods for the chemical analysis of sewage markers in soil and water in a simulated real life scenario (case study).
4. Learned how to use the above data to calculate the likely contributions of sewage to soil and water.
5. Learned how to write a professional scientific report.

Background Context

Safe and acceptable disposal of human sewage waste is an ongoing challenge for water engineers and indeed for society generally.

In the U.K. sewage treatment has, for some time, typically involved separation of the aqueous and solid phases. This is accomplished by mechanical screening, followed by settling and anaerobic digestion of the solids. The aqueous phase, sometimes after further treatment, is then typically discharged to rivers. The assimilation into UK law of the 1990 OSPAR (Oslo-Paris) Convention and the 1991 EC Urban Waste Water Treatment Directive banning offshore sewage-sludge dumping in UK coastal waters (which took effect from 1 January 1999) effectively banned disposal of the solid phase at sea, so since 1999, the de-watered solids (sometimes after so-called advanced or enhanced treatment with lime or heat), now known as 'biosolids', are typically spread onto agricultural land, landfilled or incinerated, depending on their composition and origin.

The *Safe Sludge Matrix* is an agreement made between Water UK, representing the 14 UK Water and Sewage Operators, and the British Retail Consortium (BRC) representing the major retailers and including inputs from the Environment Agency (EA), former Department of Environment Transport and Regions and former Ministry of Agriculture Fisheries and Food. The negotiations were

managed by the agricultural advisory service, ADAS, and followed a year of intensive consultation, including discussions with other interested parties such as the National Farmers Union, Country Landowners Association, food manufacturers and food processors. This agreement affects all applications of sewage sludge to agricultural land and this came into force on 31st December 1998. The provisions of the agreement were incorporated into legislation in the Sludge (Use in Agriculture) Regulations and in the Code of Practice for Agricultural Use of Sewage Sludge, both revised during 2001. The *Safe Sludge Matrix* forms the basis of the agreement and consists of a table of crop types, together with clear guidance on the minimum acceptable level of treatment for any biosolids-based product which may be applied to that crop or rotation. The *Safe Sludge Matrix* enables farmers and growers to continue to utilise the beneficial properties in sewage sludge as a valuable and cost effective source of nutrients and organic matter.

As from 31st December 1999, all untreated sludges were banned from application to food crops. The end date for the use of untreated sewage sludge on agricultural land used to grow non-food crops was 31st December 2005. The surface spreading of conventionally treated sludge on grazed grassland was banned from the 31st December 1998. Conventionally treated sludge can only be applied to grazed grassland where it is deep injected into the soil. Conventionally treated sewage sludge can be applied to the surface of grassland or for forage crops such as maize, which will subsequently be harvested, but there can be no grazing of that land within the season of application (i.e. it is not permissible to graze any grass regrowth or aftermath in the season that the sludge was applied). More stringent requirements apply where sludge is applied to land growing vegetable crops and in particular those crops that may be eaten raw (e.g. salad crops). Conventionally treated sludge can be applied to agricultural land which is used to grow vegetables in the rotation, provided that at least 12 months has elapsed between application and harvest of the following vegetable crop. Where the crop is a salad which might be eaten raw, the harvest interval must be at least 30 months. Where enhanced treated sludges are used, a 10 month harvest interval applies.

In the U.K. about 500,000 tonnes per annum of dry biosolids is applied to land used in agriculture, of which about 9% were applied to soils in the south west U.K. in 2000 (latest available public figures). Mean application rates are usually about 3 tonnes of dry biosolids per hectare to arable and pasture land.

Session 1

In the present fictitious case study, fishermen fishing a local river in the south west U.K. have complained (e.g. Figure 1) to the Environment Agency (EA) that fish caught in the area have a sewage-tainted taste and smell. In telephone conversations with the EA the fishermen suggest that the source of the alleged pollution may be illegal treatment of a local field adjacent to the river (Field B; Figure 2) with biosolids (solid sewage). However, when contacted by the EA, the water treatment company who dispose of the biosolids and their contracted spreading operator, claim that only field A, which is not adjacent to the river, has been treated, as per their permit for sewage disposal.

Figure 1

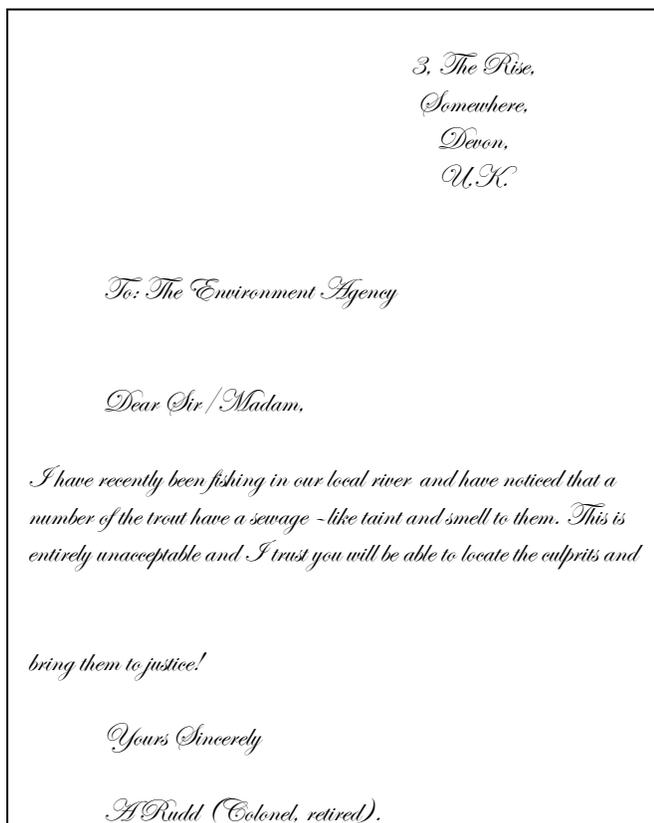
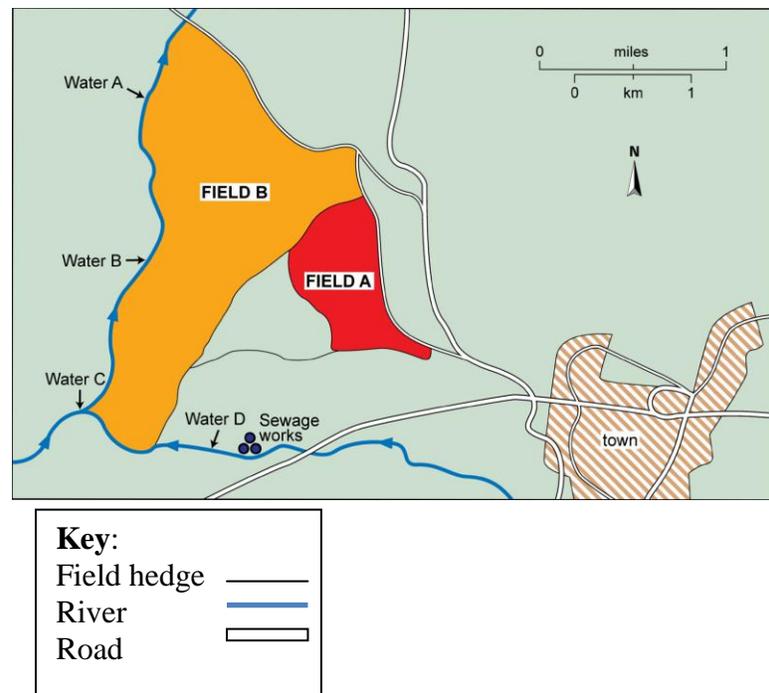


Figure 2



Class discussion

Discuss the background information to this case study using Figure 2 and suggest:

- (a) Some possible explanations of the apparent problem.
- (b) A general experimental approach to solving the problem.

Post Laboratory Exercise

Using Table 1 as a guide, conduct a literature survey of those organic chemicals (and other proxies), which have been proposed previously as markers of sewage pollution. Summarise your findings by completing the table, which you should e-mail to your tutor at least one day before the next session.

Session 2

Pre-Laboratory Exercise

As a class, discuss the amalgamated data resulting in the table of methods found in the scientific literature which have previously been found useful as markers or proxies of sewage pollution.

<i>Proxy or Marker</i>	<i>Structure*</i>	<i>Excretion rate in H. sapiens*</i>	<i>Typical concentration in sewage*</i>	<i>Physical data (e.g. solubility)*</i>	<i>Limit of detection</i>	<i>Advantages</i>	<i>Disadvantages</i>	<i>Key references</i>
Coprostanol								
Bile acids								
Aminopropanone								
Uric Acid								
Vitamin E acetate								
Enteric viruses								
Faecal coliforms								

Table 1. Literature data for organic chemical sewage proxies (and others). * Where applicable.

After further discussions you are engaged by the Environment Agency to obtain analytical chemistry data for four samples (A-D) of filtered water from the river and for soils from fields A and B (Figure 2). You decide to measure the concentrations of two organic chemicals which have been proposed previously as sewage markers; you decide to determine **uric acid** concentrations in water samples A-D and the concentration of **vitamin E acetate** in field soils A and B.

Uric acid (Figure 3a) is actually a member of the group of heteroatomic organic chemicals known as purines. Uric acid is a nitrogenous waste product, particularly of reptiles and birds, but also of humans. Average excretion amounts for an adult human are less than $600\text{-}700\text{ mg day}^{-1}$ (Stapleton, 2005). The parent compound is virtually insoluble in water, but it forms a sodium salt, which is 15 times more soluble, so the concentration of the sodium salt can be determined. A convenient method for this determination without prior purification uses high pressure liquid chromatography (HPLC) with ultraviolet (UV) detection (Brown *et al.*, 1982). The method involves comparison of the data obtained with those obtained for calibrated solutions of known concentrations of uric acid (sodium salt).

For the soil samples the concentrations of **vitamin E acetate** (also called α -tocopheryl acetate; Figure 3b) are required. Vitamin E acetate is not a natural substance but it is added to foodstuffs as an anti-oxidant. It passes through the human body partially undegraded. Thus it can be used as a sewage marker (Eganhouse and Kaplan, 1985). The best method for determination of vitamin E acetate in soils involves analysis of extracts by gas chromatography-mass spectrometry (Eganhouse and Kaplan, 1985) but a convenient laboratory method which you may use instead involves purification of the soil extracts (provided) by thin layer chromatography (TLC) and quantification of vitamin E acetate in the purified samples by measuring the UV absorption intensity of solutions of the purified material dissolved in cyclohexane and comparing with those obtained for calibrated solutions of known concentrations of vitamin E acetate.

The farmer, Mr Giles, who owns the fields, has also agreed that The Environment Agency could assess the biomass in soil samples from the fields and The Environment Agency have provided you with the data and calculated the Shannon-Weiner indices (Table 3). The latter are a measure of species diversity and biomass. Your task is to answer the question: Is there any evidence of sewage application to field B, and if so how much sewage was applied? Is this likely to be associated with the tainting of the fish or are other factors important, and if so, which? You should present your evidence in a clear and convincing manner in a professional report.

a

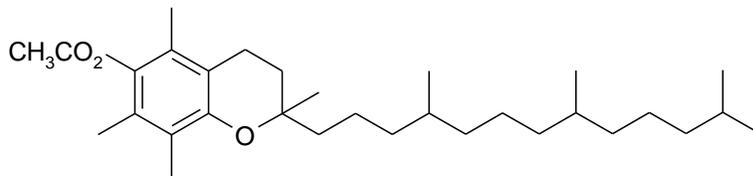
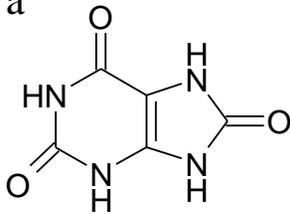


Figure 3

Experimental

Instrumentation

For examination of uric acid in water, an isocratic HPLC system with a ConstaMetric 3200 solvent delivery system, Rheodyne injector, Hyperclone 120 Å C₁₈ HPLC Column (15 cm x 4.6 mm, 5 µm), Merck Hitachi L7420 UV-VIS Detector (290 nm) and a Merck Hitachi D2500 Chromato Integrator are used. Mobile phase: 0.02% phosphoric acid in water. Injection size 100µL. Flow rate 1.2 mL min⁻¹.

There may be some variation in the HPLC equipment, so you should record all the details of the instrumentation that you actually use.

UV spectrophotometry is conducted on a Hewlett Packard (now known as Agilent) 8453 diode array detector. The instrument irradiates at 190-100 nm wavelengths using deuterium and tungsten-halogen lamps, but typically a PC is used to display spectra from 230-330 nm.

There may be some variation in the UV equipment, so you should record all the details of the instrumentation that you actually use.

Other

UV lamp for TLC plate visualisation (if silica without fluorescent indicator is used as stationary phase).

Nitrogen blow down.

Reagents

These will normally be prepared for you in advance.

1. Vitamin E acetate solution (5% v/v in dichloromethane) for TLC reference.
2. Vitamin E acetate solutions in cyclohexane for UV calibration (100, 175, 200, 275, 300, 400 µg mL⁻¹).
3. Uric acid (sodium salt) solutions for HPLC-UV calibration (0, 2000, 4000, 6000, 8000, 10,000 µg L⁻¹).
4. Dichlorofluorescein spray solution dissolved in methanol (0.2%)

Apparatus

Per student:

Auto pipette and tip (0.1mL)

Beaker (25 mL)

Drawn out melting point tubes (for use as capillaries for TLC spotting)

Aluminium foil

Spatula

Pasteur pipette plug with de-fatted cotton wool

Boss and clamp

Retort stand

Small plastic funnel

Vials (2 mL)

Volumetric flask (25.00 mL)

Pasteur pipette and teat

TLC plate (20 x 20 cm, silica HF254 x 0.25mm)

TLC tank

TLC solvent mixture (mobile phase) 100 mL hexane (95%): diethylether (5%)

Dichloromethane

Cyclohexane

Water samples A, B,C,D (100 mL each)

Soil extracts A, B (typically 20 mg). Amount of original soil must be stated.

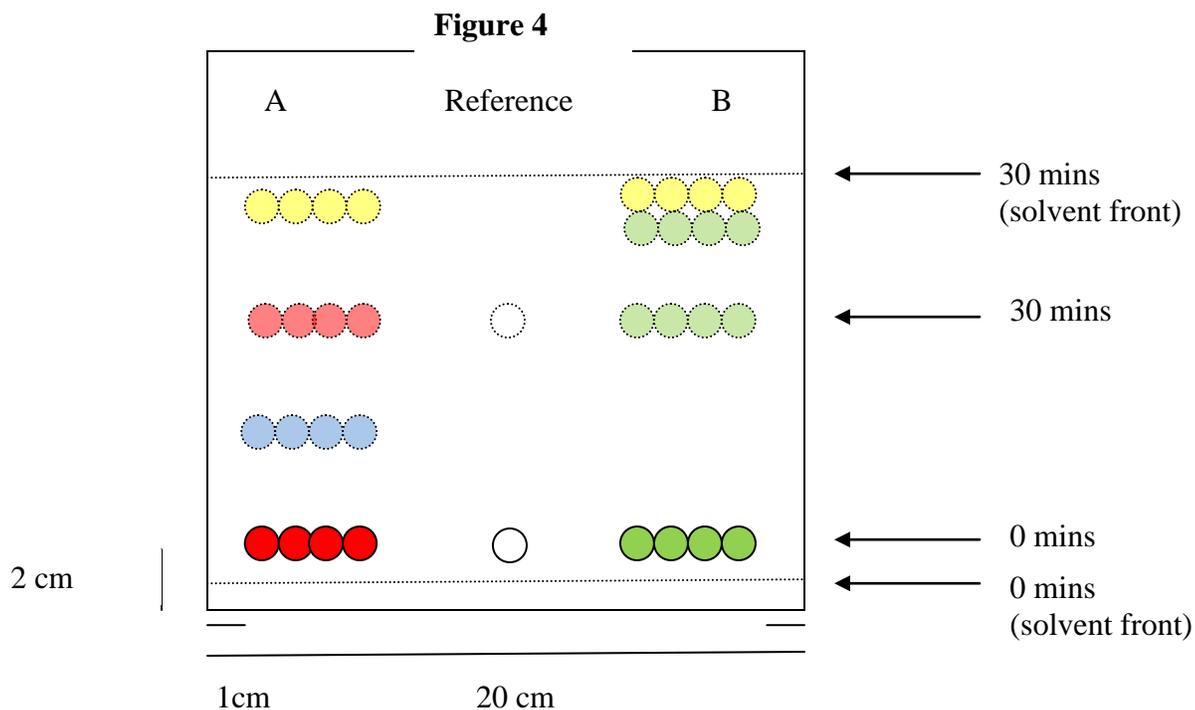
Procedure

Determination of vitamin E acetate in soil extracts from fields A and B:

You are provided with extracts of organic matter from the soils of fields A and B. These originate from the Soxhlet extraction of 200g each of dry soil A or B with refluxing dichloromethane. These extracts (typically about 20 mg each) will contain many organic compounds.

In order to isolate vitamin E acetate you need to remove many of the other compounds, or use a determination method which is specific to vitamin E acetate (or both). You can use various forms of chromatography to purify the crude extracts and then ultraviolet absorption spectrophotometry at a wavelength specific to vitamin E acetate to measure the amount present. To do the latter you will first need to examine a number of solutions (calibration solutions) of known vitamin E acetate content.

Isolate a fraction of esters (vitamin E acetate is an ester) from the crude soil extracts by thin layer chromatography (TLC). Dissolve all of each extract from soil A or B (ca 20 mg) in about 100 μ L of dichloromethane. Apply all of each sample, using a drawn out capillary glass tube, in a series of small conjoined spots, to about 2 cm above the bottom and 1 cm in from the edge of a 20 x 20 cm TLC plate coated with a thin layer of silica (Figure 4). Take care not to touch the silica with your hand or sleeve. Mark the top of the plate with an 'A' or 'B' so you know which sample is which (or use a different TLC plate for the second sample if enough are available).



Now apply a spot of a concentrated sample (5% v/v in dichloromethane) of pure vitamin E acetate to the bottom centre of the plate in line with the lines of spots of samples A and B (Figure 4). This will act as a reference for the position of the likely band of esters containing vitamin E acetate in samples A and B (if present) once the chromatogram has been developed. Now carefully transfer the silica plate to a chromatography tank containing 100 mL of hexane: diethylether (5:5) and lined with chromatography paper, taking care to allow the solvent (mobile phase) only to ascend by capillary action and not to wash off any of the sample spots. Place a top on the tank and allow it to develop until the solvent reaches about 2 cm from the top of the plate (usually about 30 minutes). Remove the TLC plate carefully and carefully mark the position of the solvent front with a drawn out glass capillary; then place the plate in a fume hood in order that the solvent may evaporate. Place the dry plate under a UV lamp in a dark cabinet and (ensuring that you are wearing safety spectacles), observe and mark the position of any obvious spots or lines. Since vitamin E acetate has a chromophore it absorbs in the UV, so it may be visible. Now stand the TLC plate against a protective cardboard screen and lightly spray the TLC plate with a solution of dichlorofluorescein in methanol. Allow the plate to re-dry and when dry examine it again (ensuring that you are wearing safety spectacles) under the UV light and mark any additional lines or spots. Non UV absorbing compounds will now quench the fluorescence of the spray and be made visible. These will include compounds such as hydrocarbons (usually observed at the solvent front).

With a ruler, starting at the midpoint of the original spots, measure the distance travelled by each of the spots and lines of spots in your developed chromatogram and also of the distance travelled by the solvent front. The distance travelled by each component divided by the distance travelled by the solvent front (the latter is usually about 17 cm) is known as the retardation factor (R_f). Record these data for your unknown spots and lines and for the known reference vitamin E acetate.

Now carefully scrape off any lines of spots in sample A (and next sample B) which you think is possibly vitamin E acetate. The silica powder (take care not to inhale this fine powder) from the relevant region should be scraped with a spatula onto a small 'boat' of folded aluminium foil. The silica should then be carefully transferred to a Pasteur pipette blocked with de-fatted cotton wool using a small plastic funnel. The pipette should be held to a retort stand with a boss and clamp. Take care not to over tighten the clamp. The analyte (suspected vitamin E acetate) can now be eluted into a preweighed (ca 5mL) glass vial with dichloromethane. The process is repeated using clean apparatus for sample B. The dichloromethane is now removed from eluted samples A and B by evaporation to dryness in a fume hood under a controlled stream of dry nitrogen. The dry vials are re-weighed. Each sample is now dissolved in a small amount of cyclohexane and the whole of each dissolved sample transferred to a 25.0 mL volumetric flask and made up to the mark. Each sample is now examined by UV spectrophotometry under the conditions listed above, the spectra recorded and the absorbance at 285 nm is recorded. The calibration solutions for 100, 175, 200, 275, 300 and 400 $\mu\text{g mL}^{-1}$ solutions of vitamin E acetate in cyclohexane are also examined and the absorbance at 285 nm recorded. A graph of the response

of the UV detector versus the concentration of vitamin E acetate should be plotted and used to determine the concentration of vitamin E acetate in the 25 mL solutions of samples A and B.

Post Laboratory Exercise

Summarise all of your data in a table. Send this table to your tutor.

Session 3

Pre- Laboratory Exercise

Discuss the summarized class data. Can you begin to conclude anything about the treatment of the fields with sewage?

Determination of uric acid (sodium salt) in river samples A-D:

Uric acid (sodium salt) may be determined directly in water by the HPLC-UV method of Brown *et al.*, (1982). Use the HPLC system described above (Instrumentation) as instructed, to examine uric acid (sodium salt) solutions for HPLC-UV calibration containing 0, 2000, 4000, 6000, 8000, 10,000 $\mu\text{g L}^{-1}$ uric acid (sodium salt). Construct a suitable calibration graph by plotting response versus concentration. Now examine each of the river water samples A-D in triplicate and use the calibration graph to deduce the concentration of uric acid in each sample as a mean \pm one standard deviation.

End Laboratory Exercise

Summarise all of your data in a table. Discuss as a class, all of the data (i.e. for vitamin E acetate and for uric acid).

Independently, using the class data but emphasising your own data, calculate the concentrations of vitamin E acetate in soil A and in soil B in $\mu\text{g g}^{-1}$ dry soil and $\mu\text{g g}^{-1}$ wet soil (soil moisture content is given in Table 2). Show clearly all your calculations. A table summarising all of the data may be useful. Conduct suitable statistical tests on the data to emphasise any similarities or differences between the fields. Use the concentrations of vitamin E acetate and the known sewage biosolids application data and field sizes (Table 2) to calculate the amount of sewage (if any) which appears to have been applied to field B. Integrate these findings with the measurements of biomass and the Shannon-Weiner diversity indices (Table 3) and of uric acid made in the river waters and use the map (Figure 2) to deduce the likely sewage contamination scenario(s). Conduct suitable statistical tests on the uric acid data to emphasise any similarities or differences between the sites and between your data and those of the whole class. Write a professional report comprising a front page summary or abstract, an introduction, description of methods, results including figures such as chromatograms and graphs and discussion, calculations/statistics and conclusions.

Map (Figure 2) courtesy of the University of Plymouth Cartography Unit. This shows a fictitious location. The field data are also fictitious.

Table 2. Data for fields A and B shown in Figure 2.

Field Code	A	B
Ordnance Survey Area (acres)	8.1	22.2
Workable area (acres)	7.3	15.6
Biosolids amount spread (m ³ dry solids per yr)	100	0
Biosolids type	Limed	Limed
Soil type*	541B	541B
Soil moisture content	30%	20%
Soil bulk density (dry)	1g cm ⁻³	1g cm ⁻³

If you wish, you may assume sewage is ploughed into a depth of 25 cm and that sewage weighs 1g per cm³.

*Soil classification of the *Soil Survey of England & Wales, Lawes Agricultural Trust*, published by Ordnance Survey (clayey loam).

Table 3. Biological data for soils from fields A and B

	Field A	Field B	Control
Biomass (g m ⁻²)	3.84±0.78	14.39±3.02	1.47±0.29
Shannon-Weiner Diversity index	1.45	0.087	2.25

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COSHH ASSESSMENT

Assessment No. 0

Assessment Date 00/00/0000

Chemicals – Hazardous in quantities used in experiment	
Hexane	Highly Flammable. Harmful by ingestion, inhalation and skin contact. There is evidence of reproductive effects.
Ethanol	Highly Flammable. Irritating to the eyes. Intoxicating if inhaled or ingested. Evacuate if spillage exceeds 700 mL.
Chemicals – Hazardous in other circumstances	
Hexane	Evacuate if spillage exceeds 150 mL.
Dichloromethane	Harmful by inhalation. Extremely irritating and damaging to the eyes. Has been found to cause cancer in laboratory animals. May cause mutagenic or teratogenic effects. Evacuate if spillage exceeds 80 mL. Short term exposure limit (STEL) 15 minutes at 300 parts per million (ppm).
Cyclohexane	Extremely Flammable. Irritating to the skin, eyes and respiratory system. Vapour can be narcotic in high concentrations. Assumed to be an irritant and narcotic if ingested. Evacuate if spillage exceeds 400 mL. STEL 15 minutes at 300 ppm.
First aid for any of the above chemicals	
Eyes	Irrigate thoroughly with water for at least 10 minutes. OBTAIN MEDICAL ATTENTION.
Lungs	Remove from exposure, rest and keep warm. In severe cases OBTAIN MEDICAL ATTENTION.
Skin	Wash off thoroughly with water. Remove contaminated clothing and wash before re-use. In severe cases OBTAIN MEDICAL ATTENTION.
Mouth	Wash out mouth thoroughly with water and give plenty of water to drink. OBTAIN MEDICAL ATTENTION.

ADDITIONAL CONSIDERATIONS: Wear nitrile gloves to handle solvents. Avoid inhalation of silica powder. Wear safety spectacles throughout and especially when viewing TLC plates under UV light. Additional information is available on request.

Author	Simon Belt, Steve Rowland
Title	Sewage Case Study
Classification	Laboratory Manuals - Chemistry
Keywords	ukoer, environmental, analytical, biomarkers, water quality, chemistry
Description	Individual lab sheets - Student
Creative Commons Licence (url)	http://creativecommons.org/licenses/by-nc-sa/2.0/uk/
Language	English
File size	400 kB
File format	pdf