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THE DETERMINATION OF ALKYLPHENOLS IN NATURAL WATERS BY SPE-HPLC-FLUORESCENCE DETECTION

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A thesis submitted in partial fulfilment of the requirements of the Nottingham Trent University for the degree of Doctor of Philosophy

This research was carried out in collaboration with the Scottish Environment Protection Agency

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I would like to dedicate this thesis to my gran and her sister, I wish they were here to see it completion.

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ABSTRACT

Nonylphenol (NP) and octylphenol (OP) have been identified as potential endocrine disrupters with the ability to induce reproductive deformities in a number of organisms. The main source of these compounds into the aquatic environment is through industrial and sewage effluent discharge. A method for their analysis has been developed making use of normal phase high performance liquid chromatography coupled with fluorescence detection.

The extraction of these compounds from river and estuarine water was developed using solid phase extraction with a C_2 packing sorbent. The complete method was validated using spiked de-ionised water and the limit of detection calculated to be 0.13 μg / l (ppb). A survey of the Forth Estuary in May 2000 found alkylphenol concentrations to be below the limit of detection though the results from a survey carried out in August of the same year produced concentrations ranging from < 0.13 – 1.90 μg / l for nonylphenol and < 0.13 – 1.28 μg / l for octylphenol.

Concentrations of nonyl – and octylphenol have been determined for suspended solids and sediment from the Forth Estuary. NP and OP from the solid material was extracted into iso-hexane over a period of 6 hours using soxhlet apparatus. The extract was subject to solid phase extraction using an amino packing sorbent prior to analysis. The limit of detection for the sediment method is calculated to be 10.2 μg / kg (ppb) for both NP and OP. The surveys of the Forth Estuary for both May and August produced sediment results ranging from < 10.2 – 152 μg / kg for nonylphenol and < 10.2 – 87.4 μg / kg for octylphenol. The limit of detection for the suspended solid work is calculated to be 0.08 μg / l (ppb). The resulting concentrations from the May and August survey are 0.09 – 0.26 μg / l for nonylphenol and <0.08 – 0.26 μg / l for octylphenol. The results are consistent with a river estuary that is contaminated, though not grossly polluted by NP and OP.

Although these results show NP and OP to be below toxicity thresholds for many aquatic organisms, the scope for their bioaccumulation within aquatic species means a chronic, rather than acute impact from NP and OP inputs might be anticipated.

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Chapter 1: Introduction

1.0 Introduction

In recent years there has been growing concern over compounds that exhibit oestrogenic activity (xenoestrogens). The effects of these compounds include deformities in both the male and female reproductive tracts, as well as links with reduced sperm quality [Toppari *et al.*, 1996]. Many organisms have exhibited evidence of exposure to such xenoestrogens, including fish, reptiles and humans [Jobling *et al.*, 1996, Palmer and Palmer, 1995, Colborn *et al.*, 1996]. Worryingly the results of xenoestrogen exposure are thought not to be the result of isolated pollution incidences, but rather daily exposure to these compounds.

The identification and measurement of specific oestrogenic substances in the environment is of great importance, especially if regulatory controls on such substances are to be introduced. The majority of the research undertaken so far has concentrated on the biological effect of these compounds but little has been developed for the analysis and identification of the xenoestrogens. This study develops a reliable and efficient method for the analysis of alkylphenols (known xenoestrogens), in both river and estuarine waters and sediments, allowing for subsequent assessment of environmental concentrations and pathways of selected xenoestrogens in river and estuarine systems in the U.K.

1.1 Xenoestrogens

The compounds that have been identified as xenoestrogens include phthalate esters, tributyl tin, polychlorinated biphenols (PCBs) and alkylphenols [Toppari *et al.*, 1996]. The xenoestrogens are used extensively as plasticizers, detergents and employed in paint and pesticide formulation, they are therefore ubiquitous in the environment. Many of these compounds have shown oestrogenic activity both *in vivo* [Chapin *et al.*, 1999] and *in vitro* [Mueller and Khim, 1978]. The alkylphenols (known xenoestrogens) are 10^3-10^4 less potent than 17β-oestradiol (a naturally occurring oestrogen) [White *et al.*, 1994], and this would suggest that they were of no threat to the environment.

However;

- 17β-oestradiol can initiate a response within a cell at the ng / l level,
- xenoestrogens are present in larger quantities than naturally occurring hormones,
- they have the ability to accumulate in different matrices including fatty tissue [Duarte-Davidson and Jones, 1996],
- the xenoestrogens are not deactivated by binding to blood proteins unlike the natural oestrogens, so they circulate in the body for longer.

This would suggest that the biological effects of the xenoestrogens are potentially of significance to both human and other species [Colborn *et al.*, 1996, Kloas *et al.*, 1999].

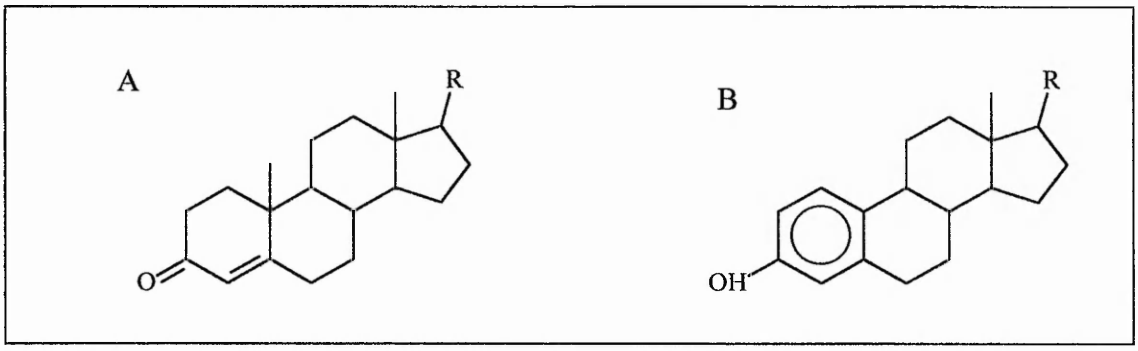

Figure 1.1 *Structures of the sex hormones (a) testosterone (R=OH) and (b) oestradiol (R=OH) and oestrone (R=O).*

Oestrogens are one of the classes of steroid hormone, along with androgens, that are classed as the sex hormones of both the male (Fig. 1.1a) and female (1.1b) in many species. The structures of the female oestrogens, namely oestradiol and oestrone are unique as they are the only steroids to posses an aromatic ring. In the cells of the body, the hormones are site specific and are activated on attachment to appropriate oestrogen receptor sites that are found within the cell. Experimental work has shown the decrease in the number of receptor sites available to 17β-oestradiol in the presence of certain synthetic compounds [White *et al.*, 1994].

The compounds under investigation in this study (the alkylphenols) possess the ability to initiate a similar response within a cell as that of oestrogen, for example, they act *via* oestrogen receptors. The oestrogen receptors are found in cells throughout the human body [Mueller and Khim, 1978], the receptors are also found in most if not all mammals and a wide variety of amphibians.

1.1.1 The Endocrine System

The endocrine system is one of the three main systems of the human body and is operated by chemical signals known as hormones. The group of hormones of interest in this study are the steroids, namely oestrogen and testosterone. These hormones are responsible for development, including sexual differentiation and maturation, as well as carbohydrate metabolism, salt and water balance and lipid deposition. The hormones are synthesised in special cells, these cells may form a specific organ, e.g. adrenal gland or they may be single cells within an organ, e.g. Leydig cells within the testes. These hormones are produced from specific sites that are situated in various parts of the body as shown in Fig. 1.2.

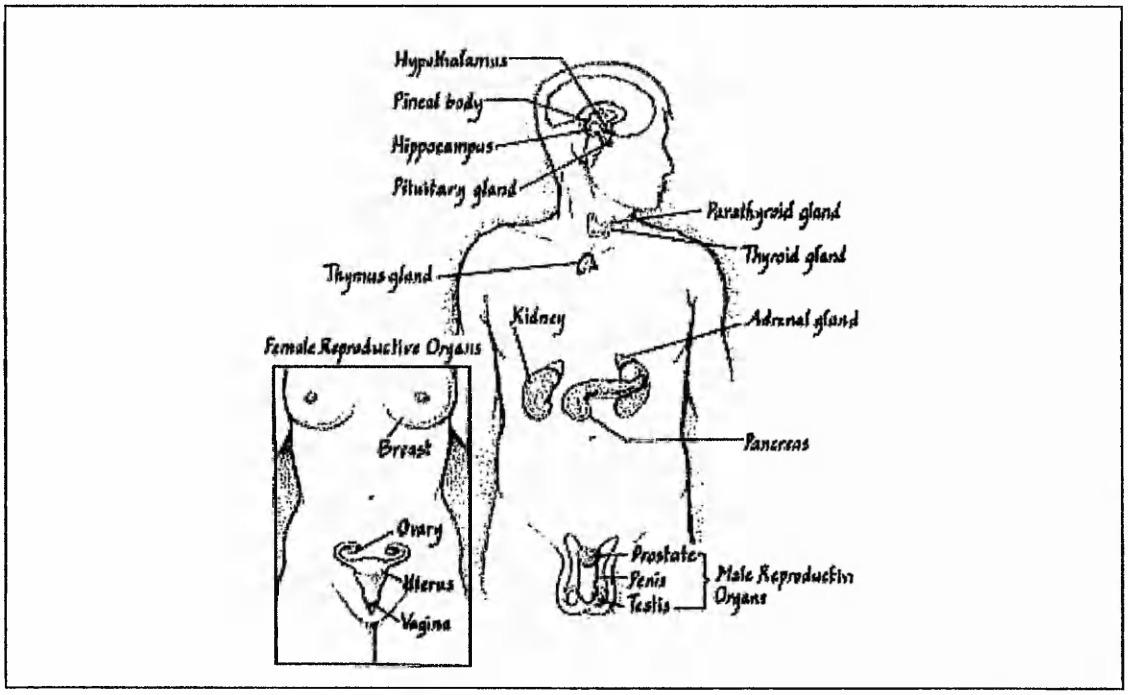

Figure 1.2 *The sites of hormone production in the body (www.wwfcanada.org/)*

Unlike other hormones, the sex hormones are synthesised when needed and released directly into the blood stream, the molecules may be found in a free state or attached to specific proteins. The free molecules are more readily diffused into the tissue and therefore equilibrium is formed in the blood between the free hormone and that, which is bound to the specific proteins. On entry to the tissue they bind to specific regions of DNA. A signal is produced by the DNA and activates specific genes, which ultimately lead to a response in the target cell.

1.1.2 Hormone disrupters

The normal response of a hormone is shown schematically in Fig. 1.3a, with the hormone fitting into the receptor and initiating a response for gene expression. However, the hormone disrupters interact with the receptors where they may either mimic the hormone giving a similar response to that shown in Fig. 1.3a, or they may block the receptor site to prevent the correct hormone signal (Fig.1.3b).

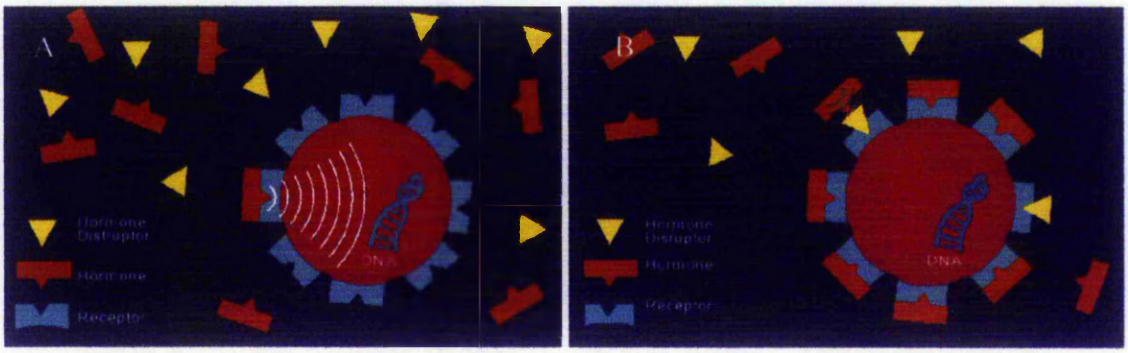

Figure 1.3 *The various interactions at the oestrogen receptor site. A) Formation of the hormone–receptor interaction and response initiated. B) Blocking of the receptor site by the endocrine disrupter [www.wwfcanada.org].*

A hormone mimic may either exaggerate the response initiated by the natural oestrogens or a response may be generated at the wrong stage of development resulting in a change in biological effect. A hormone blocker will attach to the oestrogen receptor but no signal will be generated and the site will be blocked from use by the natural oestrogens as shown in Fig. 1.3 b. There have also been examples of endocrine disrupters that have the capability of stimulating the formation of more receptor sites, this will again lead to amplification in hormone signal [www.wwfcanada.org].

1.1.2 Effects of the endocrine disrupters

Alkylphenols are able to displace 17β-oestradiol from the oestrogen receptor [Mueller and Khim, 1978] with relative binding affinity in comparison to oestradiol of 0.031 for nonylphenol and 0.015 for octylphenol [Blair *et al.*, 2000]. Soto *et al.*, (1991) discovered that nonylphenol leaching from polystyrene could induce breast tumour cell proliferation (an effect usually induced by oestrogen). Further work in this area showed octylphenol to have a greater potency than nonylphenol [Soto *et al.*, 1995]. Nonylphenol has been subject to *in vitro* toxicity testing and results suggest that at low concentrations nonylphenol inhibits ATP (adenosine triphosphate) synthesis in mitochondria by disruption of the mitochondrial membrane [Bragadin *et al.*, 1999], resulting in a decrease in energy available to the cell.

1.1.2.1 Effects on humans

Endocrine disrupters have been linked to the increase in reproductive disorders in both male and female of a variety of species, male deformities include cryptochidism (maldescent of the testis), hypospadias (the urinary opening found on the underside of the penis) [Toppari *et al.*, 1996], as well as declining sperm count [Montagnani *et al.*, 1996, Jensen *et al.*, 1995]. Studies with mice exposed *in utero* to octylphenol demonstrated reduced sperm production and efficiency at 2 ng / g [Saal *et al.*, 1998]. Colborn *et al.*, (1996) found that children whose mothers ate contaminated fish from the Great Lakes (USA / Canada) on a regular basis during pregnancy were of a lower birth weight, were found to have weaker reflexes, jerky movements and impaired cognitive function. As these children matured they also showed signs of hyper-reactivity and received lower scores in intelligence tests.

1.1.2.2 Effects on mammals

In vivo studies on rats looked at transgenerational effects upon exposure to nonylphenol at concentrations ranging from 200 – 2000 mg / kg, the results indicate an effect on reproduction at concentrations of 100 – 350 mg / kg / day, with lower doses of 12 – 40 mg / kg / day causing greater problems with the kidneys [Chapin *et al.*, 1999]. Other rat studies have investigated the embryotoxicity of nonoxynol - 9, the active ingredient in many contraceptive products that degrades to form nonylphenol. The *in vivo* study by Tryphonas and Buttar, (1986) concluded that nonoxynol-9 was toxic to both embryo and foetus under the conditions of the study if administered during the first week of gestation.

1.1.2.3 Effects on aquatic organisms

Endocrine disrupting effects have also been observed in aquatic organisms, mainly by the use of vitellogenin production as a biomarker of oestrogenic activity. Vitellogenin is an egg yolk protein produced by mature female fish in response to an oestrogen signal. This response can be utilised in terms of xenoestrogen monitoring of water systems, as blood samples from male or immature female fish can by tested for vitellogenin presence with relative ease. Rainbow trout (*Oncorhynchus mykiss*) were, for a three week period, exposed to nonyl and octylphenol at concentrations of 30 μg / l, as well as monitoring vitellogenin production, testis weight was also recorded [Jobling *et al.*, 1996]. A number of conclusions could be drawn from the results obtained, namely, a response can be both dose and life stage sensitive, with threshold concentrations of 10 μg / l for nonylphenol and 3 μg / l for octylphenol.

Pedersen *et al.*, (1999) investigated the effect of branching on the alkyl chain by exposure of rainbow trout to branched or linear nonyl or octylphenol over a period of nine days. The results observed a vitellogenin response with the branched alkylphenols only, with response independent of exposure route. Common carp (*Cyprinus carpio*) has also been subject to study with exposure to nonylphenol at concentrations from 1-15 μg / l over 70 days, the blood tests indicated severe anaemia though no obvious histopathological effects at concentrations above 5 μg / l [Schwaiger *et al.*, 2000].

The use of indicator species has been suggested for environmental monitoring, with coastal species of cod (*Gadus morhua*) and flounder (*Platichthys flesus*) appearing to be good candidates. Both have close contact with sediments as a result of their feeding habits and are therefore likely to be the first species to indicate a contamination problem [Goksoyr *et al.*, 1996]. For freshwater monitoring it may be more difficult to pick a single species for monitoring, though there is now a great deal of information gathered on the rainbow trout.

1.1.3 Bioaccumulation and toxicity

A number of toxicity studies have been employed on a wide range of aquatic species and under various experimental conditions. Table 1.1 gives a few examples of lethal concentration to 50 % of the population (LC50) over various timescales.

Table 1.1 *Freshwater organism toxicity data for nonylphenol (NP) and octylphenol (OP).*

Species	**LC50 (mg / l)**	**Observation time**	**Reference**
Crangon septemspinosa **(Common estuarine shrimp)**	OP-1.1	96 hour	McLeese *et al.*, 1981
Salmo salar **(Atlantic salmon)**	NP-0.3		
	NP-0.19		
Oryzias latipes **(Japanese Medaka)**	NP-1.4	48 hour	Yoshimura, 1986
Mytilus edulis **(Common mussel)**	NP –3	96 hour	Granmo *et al.*, 1989
	0.5	360 hour	
	0.14	850 hour	
Fundulus heteroclitus **(Mummichog)**	NP larval 0.204	96 hour	Kelly and Giulio, 2000
	NP embryo 5		

Servos (1999) reviewed toxicity data for nonyl- and octylphenol and their ethoxylates, a summary of the results from more than twenty two species involved in LC50 96 hour studies indicated acute toxicity to fish at concentrations of 17-3000 μg / l, invertebrates 20-3000 μg / l and 27-2500 μg / l for algae. Algae studies produced similar results to those observed in other organisms with effects on growth seen at concentrations of 25 μg / l. LC 50 results for marine organisms are displayed in Fig. 1.5.

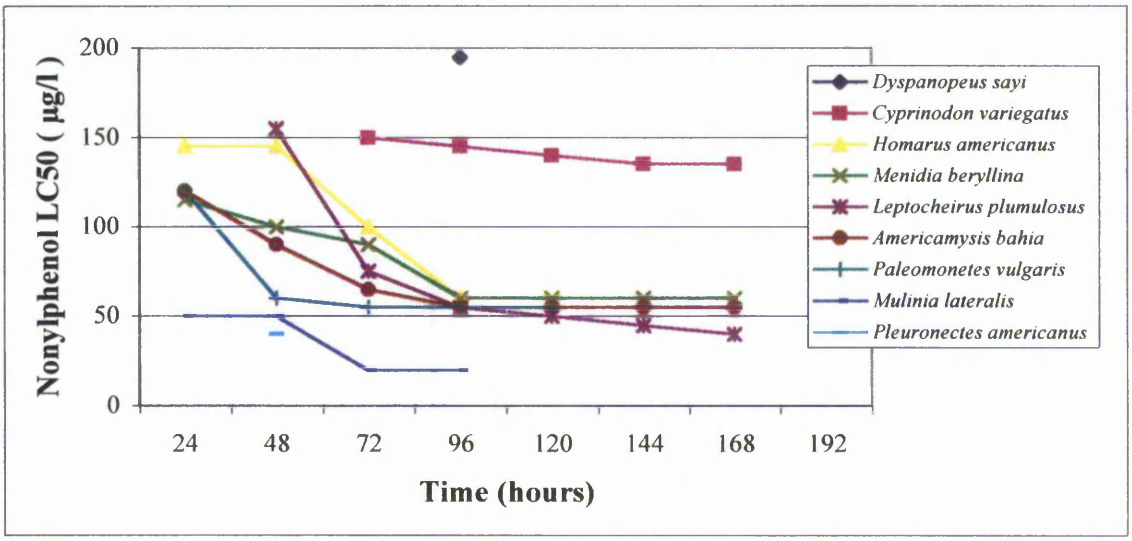

Figure 1.5 *Saltwater species LC 50 data from 24 to 168 hours, adapted from Lussier et al., 2000. The English names for the marine species are, in descending order: stone crab, sheepshead minnow, American lobster, Mississippi silverside, amphipod, mysid shrimp, grass shrimp, bivalve, winter flounder*

From these studies a number of conclusions have been drawn though there are still many unanswered questions. Tolls and Sijm (1995) observed that although hydrophobicity may play a large part in bioconcentration, other factors such as water hardness, ionic strength and dissolved organic carbon content should also be considered. They also noted that surfactant elimination from the body was independent of hydrophobicity. Biotransformation has also been observed using radiolabelled compounds, with a majority of the labelled analyte eliminated from the body 72 hours after exposure [Coldham *et al.*, 1998]. The work is encouraging in the fact that elimination is possible, however in terms of environmental application, it is unlikely that a fish will be exposed to a single xenoestrogen application due to the continuous nature of such discharges into the water system.

The majority of environmental xenobiotics are lipophilic in character and have the potential for storage and accumulation in fatty tissue [Tolls and Sijm, 1995]. A bioconcentration factor has been derived from the ratio of the analyte concentration in the organism to the concentration in the water column. Algae, fish and ducks have exhibited bioaccumulation of alkylphenols from the water column [Ahel *et al.*, 1993] as well as marine organisms such as the common mussel and shrimp [Ekelund *et al.*, 1990]. Algal bio-concentration factors (BCF) of 7700 were calculated by Ahel *et al.*, (1993), while BCFs for stickleback (*Gasterosteus aculeatus*), common mussels (*Mytilus edulis*) and European brown shrimp (*Crangon crangon*) were 1300, 3400 and 6500 respectively [Ekelund *et al.*, 1990].

Wahlberg *et al.*, (1990) dispatched a number of cages containing mussels (*Mytilus edulis*) around sewage discharge pipes at eight sewage treatment plants in Sweden. The experiment recorded concentrations of nonylphenol to be present within the range of 26 – 1100 μg / g (dry weight). They also placed a number of cages at various points around a single discharge pipe varying the direction and depth, these results suggest greater concentration in the mussels that were placed near the bottom of the water course, with concentrations ranging from 0.2 – 0.4 μg / g (wet weight). These elevated concentrations would suggest that the results might be linked to xenoestrogen-enriched sediment.

A positive relationship was observed in the bluegill sunfish (*Lepomis macrochirus*) between nonylphenol concentration in water and tissue, though there was no link between tissue and lipid content [Liber *et al.*, 1999]. The bioaccumulation factor was calculated to be 87, with tissue concentrations of nonylphenol at 0.01 – 2.94 μg / g. A study of the shorter chain alkylphenols in the Detroit River found that analyte levels in carp exceeded those found in the surrounding sediment indicating bio-concentration (Shiraishi *et al.*, 1989). When determining the toxicity of these compounds it is important to consider not only the dosage of one xenoestrogen but also the many others that are present in the environment. Therefore it is worth considering the possible cumulative or synergistic effects that may be induced by this xenoestrogenic cocktail.

1.2 Detection and screening techniques for oestrogenic activity

There are a number of screening methods available for the assessment of compound oestrogenicity. The following sections will discuss some of the screening and detection methods, dealing with biological screening, chemical detection as well as the prediction of potential endocrine disrupters using structural activity.

1.2.1 Biological screening

In vitro tests include the use of rainbow trout liver cells. They respond to oestrogenic stimulation by the production of vitellogenin and this response can be measured with relative ease *via* a blood test. *In vivo* tests on rainbow trout have been performed using this technique [Pedersen *et al.*, 1999, Harries *et al.*, 1996, Lech *et al.*, 1996], as have toads and other amphibians [Palmer *et al.*, 1995]. Breast cancer cells are also used as indicators, with cell proliferation occurring in the presence of oestrogen [Soto *et al.*, 1991]. These biological tests can indicate the presence of an endocrine disrupter but it is not compound specific, therefore analytical techniques are required to identify and quantify the individual oestrogen mimicking compounds.

1.2.2 Chemical detection

There are a number of techniques available for the chemical analysis of potential endocrine disrupters, these include, HPLC (high performance liquid chromatography)-fluorescence [Khim *et al.*, 1999], gas chromatography – mass spectrometry (GC-MS) [Long *et al.*, 1998] and electrochemical [Waters *et al.*, 1986]. These analytical methods will be discussed in more detail in Section 1.3.3 and Chapter 2.

Desbrow *et al.*, (1998) combined analytical methods with biological in an attempt to identify the compounds in sewage responsible for inducing oestrogenic responses in aquatic organisms. Chemical fractionation was employed using filtration followed by solid phase extraction. At each stage the liquor was assessed for oestrogenic activity using a yeast based screening test. C_{18} SPE (solid phase extraction) was found to retain a majority of the activity, therefore this fraction was subject to preparative HPLC on an octadecylsilane column and final analysis was performed by GC-MS. The compounds identified as those responsible for oestrogenic activity were the natural and synthetic steroidal oestrogens.

Thomas *et al.*, (1999) carried out a similar study on several UK estuaries. The water was fractionated by SPE and HPLC, with toxicity assessed by two bioassays and the toxicants identified by GC-MS. The results indicated that each estuary sampled had its own individual toxic component. The River Tyne was susceptible to chlorinated phenols, while the Tees had greater concentrations of alkylphenols and the River Mersey was subject to dieldrin pollution. These results differ from those found in the study by Desbrow *et al.*, (1998), Thomas (1999) was looking at toxicity where as Desbrow was specifically looking for oestrogenically active compounds.

1.2.3 Structural activity

There are a large number of compounds that have now been identified as environmental oestrogens, whether they are naturally occurring e.g. the phytoestrogens or synthetic e.g. pesticides. Examples of some of these compounds and their structures are found in Fig. 1.6.

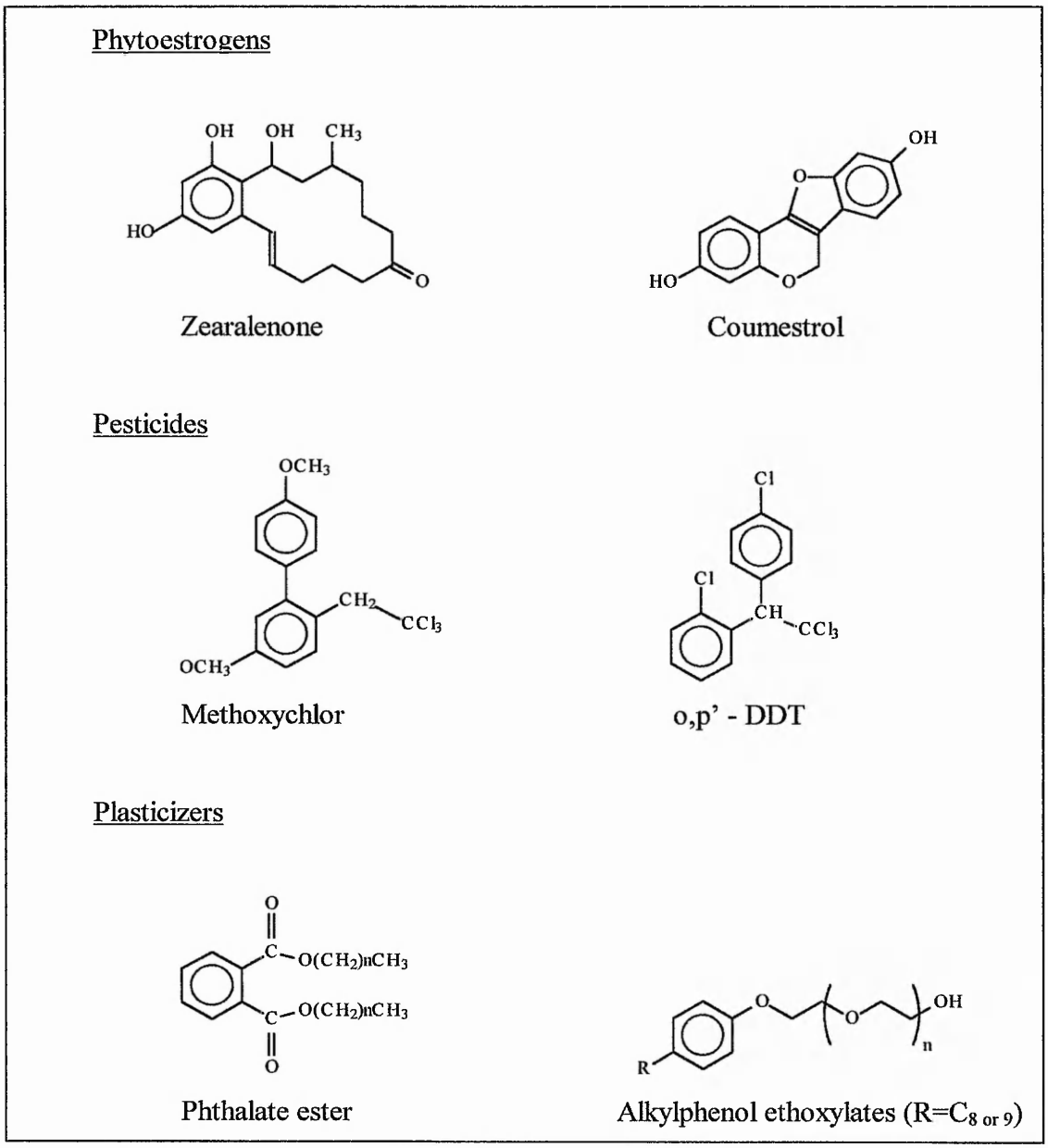

Figure 1.6 *The structures of some natural and synthetic oestrogens.*

Attempts to find a structural relationship between suspected xenoestrogens and oestrogenically active compounds have been partially successful with a majority of the compounds containing a para-substituted phenolic group [Nimrod and Benson, 1996]. Quantitative structure-activity relationship (QSAR) models have been successfully used for the prediction of relative binding affinity of selected compounds to oestrogen receptors [Tong *et al.*, 1997]. This offers a cheaper form of screening with only the compounds testing positive to the QSAR model being put forward for biological screening. Schmieder *et al.*, (2000) has used this model to predict the binding of alkylphenols to the oestrogen receptor, with the theoretical results comparing well with the practical. In time it is hoped that these models may be able to accurately predict the oestrogenic potential of unknown compounds rather than just predict the relative binding affinity.

1.3 Alkylphenols and their ethoxylates

The alkylphenols and their ethoxylates (APEs) are the main compounds of interest in this present study. They are known to exhibit oestrogenic activity in both fish [Harries *et al.,* 1996] and human cells [White *et al.,* 1994]. A voluntary ban has been implemented across Europe on the use of APEs as non-ionic surfactants in domestic cleaning products [Marcomini *et al.,* 1988], however they are still used extensively in industrial cleaners but such use is being phased out. Water quality levels for the APEs are to be established in the near future [ENDS report, 1999], though the concentrations have yet to be decided, as has the date of application [Coyle, 2001]. No such ban has yet been imposed in the United States where two thirds of these compounds for the global market are produced.

1.3.1 The manufacture and uses of the alkylphenols

Nonylphenol ethoxylates account for 80 % [Naylor, 1995] of the total APEs produced, whilst octylphenol ethoxylates represent between 15 – 20 %. Nonylphenol is produced industrially by alkylating phenol with mixed propylene trimers in the presence of an acid catalyst, with 4-octylphenol produced by phenol alkylation with diisobutylene [Maguire, 1999]. Commercial APEs are usually produced as a mixture of oligomers whose distribution is dependent on the initial ratio of ethoxylate to alkylphenol prior to reaction. The oligomeric composition indicates the surfactant properties, with some compositions being lipophilic and others hydrophilic, these characteristics dictate the stability of the emulsion and its oxidative resistance [Sun *et al.*, 1996]. The APEs are used extensively in industry, with utilisation of their surfactant properties shown in Table 1.2.

Table 1.2 *Uses of the alkylphenol ethoxylates (55 %, 30 % and 15 % of total APEs produced)*

Properties	Industry	Use
Dispersing agent	Paper	Pulp and de-inking processes
Emulsifying agent	Pesticide	Improve adhesion of toxicant to organism
Stabiliser	Plastics	Emulsion polymerisation and stabiliser in latex
Wetting agent	Textile	Good handling and rinsing characteristics
Detergent	Institutional	Hard surface and commercial vehicle cleaners
Detergent	Household and personal care products	Liquid laundry detergents, shower gel and contraceptive products

Octylphenol ethoxylates have been used more specifically for surface coatings such as brake and clutch linings as well as in the formulation of special printing inks. Other uses include anti-fatigue and plasticising agents in the rubber chemical industry [Maguire, 1999]. Nonylphenol has been incorporated into fuel as a marker for taxation in conjunction with diisobutyl phthalate.

1.3.2 Biodegradation of alkylphenols in the aquatic environment

The APEs are discharged into streams and rivers *via* industrial discharges, sewage treatment effluents and land fill leachates [Marcomini *et al.*, 1989]. Degradation occurs rapidly with the systematic breakdown of the ethoxylate chain until only the alkylphenol remains [Giger *et al.*, 1984]. A schematic diagram of the degradation pathway is shown in Fig. 1.7. As degradation of the ethoxylate chain proceeds there is a decrease in water solubility of the degradation products as well as an increase in toxicity [McLeese *et al.*, 1981, Naylor *et al.*, 1992]. The short chain APEs and the alkylphenol are found to have a greater affinity for particulate matter and therefore find an environmental sink in sediments [Isobe *et al.*, 2001] and sewage sludge [Stephanou and Giger, 1982].

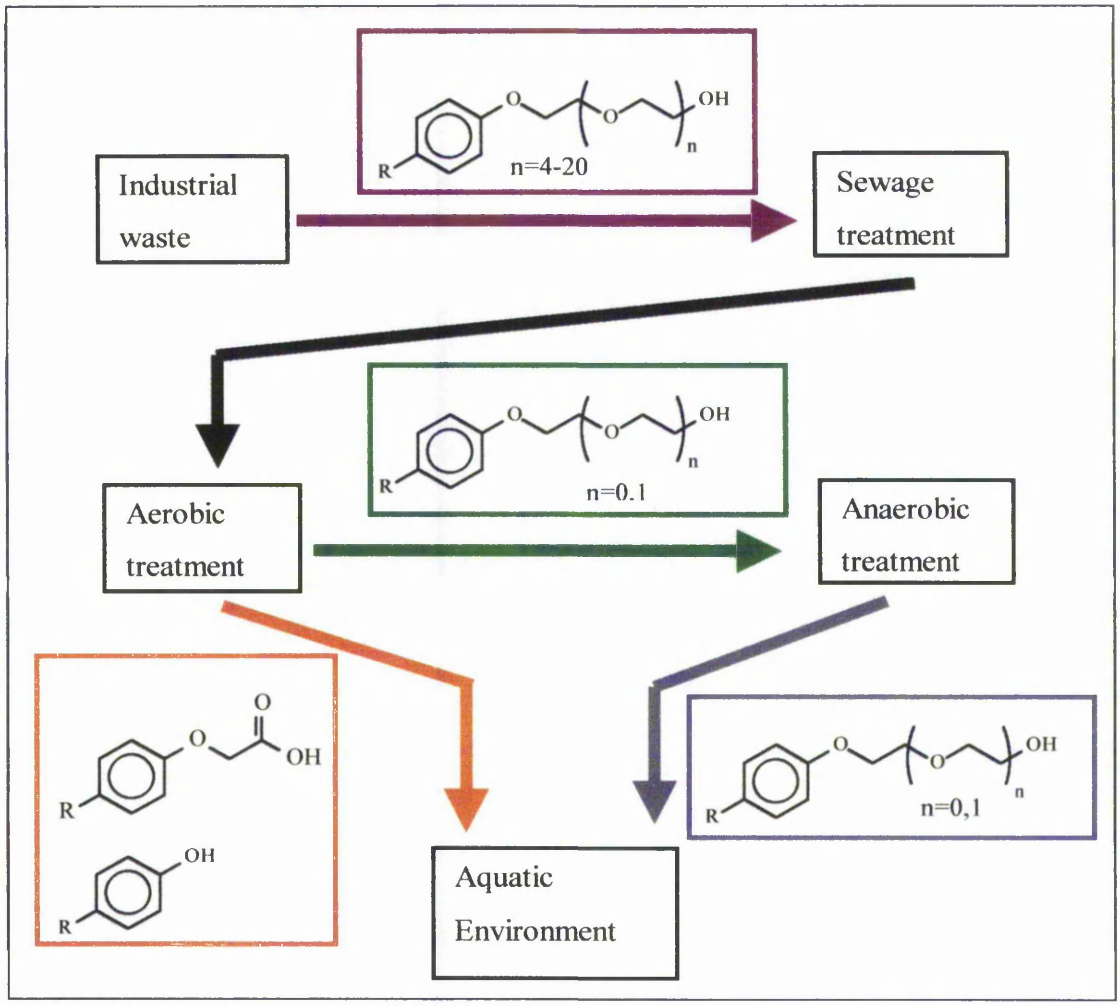

Figure 1.7 *Degradation pathway of the alkylphenol ethoxylates*

Many of the studies agree that biodegradation in the aquatic environment is dependent upon temperature [Tanghe *et al.*, 1998], with degradation significantly reduced at winter water temperatures [Ahel *et al.*, 1996]. Kravetz, 1991 found low degradation of 30 % over 28 days at temperatures of 25 °C for nonylphenol ethoxylates, whilst Tanghe *et al.*, (1998) reported total degradation of nonylphenol in activated sludge at 28 °C when spiked at a concentration of 8.33 mg / l. Rudling and Solyom, (1974) observed 50 % of the APEs in sewage effluent discharge to be degraded at a temperature of 20 °C, while at 15 °C there was no degradation reported. Ahel *et al.*, (1994) observed near complete degradation between 6 – 23 days depending on the initial concentrations, they also observed that rate of degradation was dependent on the acclimation of the bacteria to the alkylphenol ethoxylates.

A majority of these studies have been applied to artificial aquatic systems though it is possible that greater degradation in sewage treatment works (STW) will occur in comparison to lab experiments as a result of greater variation in the microbial population and nutrient content. It is also worth noting that once the APEs are discharged into the river systems from the STW a majority of the biodegradation will have already occurred, it would therefore be of greater interest for these river and estuarine studies to concentrate on the short chain ethoxylates (n=1-3) and alkylphenols.

Research carried out by Giger *et al.,* (1984) estimated that 60 % of all nonylphenol ethoxylates produced entered the sewage system, where a majority of the ethoxylates were broken down to form nonylphenol. A majority of the nonylphenol was then associated with the digested sludge and disposed of accordingly, with only a small percentage being discharged into natural waters. These degradation and discharge results will vary depending on the source of the input into the sewage works i.e., industrial or domestic. The type of treatment system used will also contribute greatly to the degradation process (Fig. 1.6). The most abundant of the nonylphenol ethoxylate (NPE) degradation products found in the water column after sewage treatment are the short chain ethoxylates, this situation is altered if the effluent is subject to secondary sewage treatment and the carboxylic derivatives show greater abundance [Ahel *et al.*, 1987]. A review by Bennie *et al.*, (1998) observed APE metabolites from Canadian sewage treatment plants to be present at concentrations below 5μg / l in the discharged effluents, though a majority of sewage sludge was contaminated at levels above 100 μg / g.

Marcomini *et al.,* (1990) observed that sediment concentrations were not affected by temperature with concentrations remaining constant throughout the year. Bennett and Metcalfe (2000) have studied the distribution of alkylphenols along a water system, with results indicating a decrease in nonylphenol concentration downstream of the point of discharge. Octylphenol concentrations follow the same trend but are generally two orders of magnitude lower. A study on the Krka River Estuary, Croatia by Kvestak *et al.*, (1994) observed a complicated distribution pattern that was dependent on the salinity profile of the estuary. Maximum concentrations were detected at the phase boundaries, between the brackish and saline water at concentrations of 1.3 – 8.9 μg / l and the surface microlayer (3.9 – 17 μg / l). There was little or no degradation of nonylphenol under the estuarine conditions.

The environmental persistence of alkylphenols have been examined recently by Heinis *et al.,* (1999), with littoral enclosures erected to evaluate distribution *in situ.* Four concentrations were assessed, 3, 30, 100, and 300 μg / l, with ten applications occurring over a period of 20 days. For the higher application concentrations of 100 and 300 μg / l, traces of 0.22 and 0.59 μg / l respectively were detected in the water 98 days after the last application prior to ice formation and by the following Spring (313 days) the levels were below the limit of detection (< 0.14 μg / l). Sorption was assessed by measuring the concentration of nonylphenol on the enclosure walls, in the macrophytic community and within the sediment. The 300 μg / l application enclosure recorded concentrations in the macrophytes to be constant at 3.26 mg / kg from day 56 – 318 post-application, though levels were below detection at day 440 (< 0.63 mg / kg).

Surface sediment concentrations from the highest application enclosure indicated an absorption maximum (27.4 mg / kg) at 48 days after the initial application. These levels decreased gradually but were still detected at concentrations of 4.9 mg / kg after day 318, this reduction may be the result of uptake by macrophytes or degradation. Core sediment samples used for the determination of biodegradation concluded that sediment-associated nonylphenol was resistant to microbial attack under the conditions of the study. This would conclude that surface sediment was more susceptible to degradation, possibly as a result of aerobic conditions.

There have been two sediment cores taken from the Tokyo Bay, Japan and assessed for pollutant concentrations by Yamashita *et al.,* (2000) and Isobe *et al.,* (2001). The Yamashita study looked at a range of pollutants within the profile of the sediment core, though the results of interest are nonyl- and octylphenol concentrations. Nonylphenol concentrations varied from < 10 – 5,540 ng / g (dry weight), the levels increased with year to a maximum concentration observed in the core section relating to the mid-1980's, from this point the concentrations stabilised and started a gradual decrease. Octylphenol concentrations were also assessed and ranged from < 10 – 190 ng / g, with results 5 – 50 times less than those of nonylphenol. The nonylphenol results are summarised in Fig. 1.8 and compared to the results of the Isobe study.

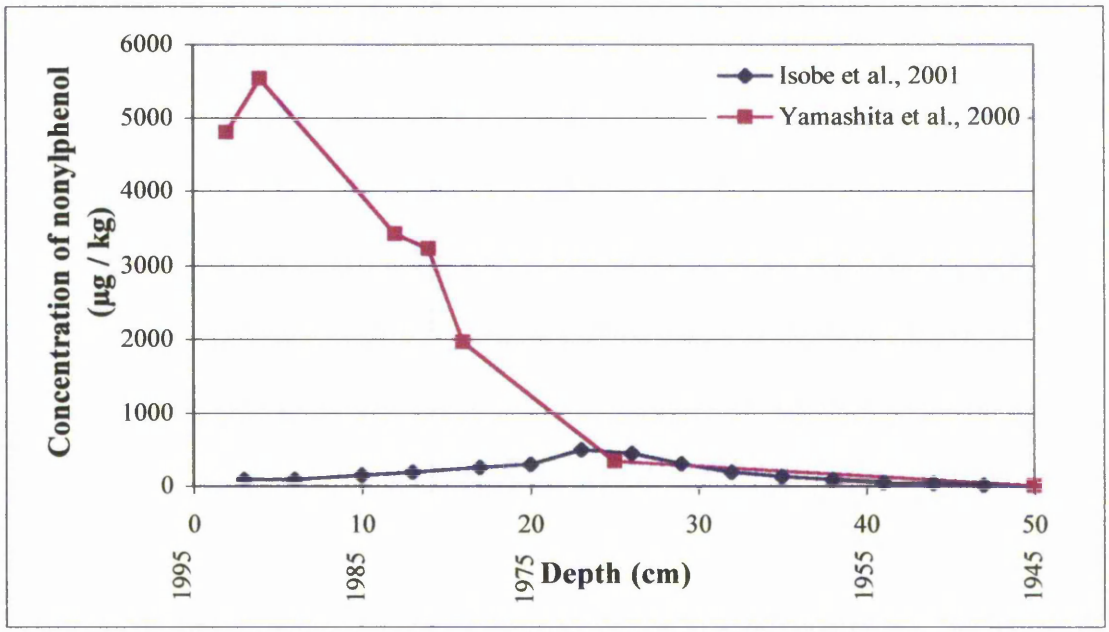

Figure 1.8 *Nonylphenol concentrations in two sediment cores from the Tokyo Bay, analysed by two different research groups and the results compared.*

The Isobe result suggests a concentration maximum that is several orders of magnitude lower than observed by Yamashita as well as being detected approximately 10 years previous. This does not correspond to AP production, which has steadily increased since production started in the late 1950's. The results would be expected to vary to a certain extent as a consequence of different sampling strategies as well as location but not to the degree observed in these results. Improvements in the sewage treatment processes may help to reduce levels but not to the extent that is reported by Isobe *et al.,* (2001).

1.3.3 Methods for the analysis of alkylphenols

A number of analytical techniques have been employed for the extraction and analysis of alkylphenols and their ethoxylates, a summary of these techniques are found in Table 1.3.

Table 1.3 *Review of extraction and detection techniques adapted from Lee, 1999.*

Compound	Sample	Extraction	Detection	Limit of detection	Reference
NPE $_{(2-6)}$	Aqueous stds.	N/a	LC-MS	160-240 ng / l	Takino *et al.*, 2000
NP	UK Estuaries Water Sediment	C_{18} SPE Ultrasonic / C_{18}	GC-MS	0.1 µg / l 0.1 µg / g	Blackburn *et al.*, 1999
NP and short chain ethoxylates	Algae, Fish and duck	Steam distillation /solvent extraction	NP-HPLC	30 µg / kg	Ahel *et al.*, 1993.
Octylphenol Nonylphenol	N/a	N/a	Silica HPLC Fluorescence	100 µg / l	Ibrahim and Wheals, 1996
Nonylphenol	US Rivers: water sediment	Steam distillation	HPLC- fluor.	0.107 µg / l 2.93 µg/g	Naylor *et al.*, 1992
NPE	Sewage effluent	Gaseous stripping	HRGC-MS	1 µg / l	Ahel *et al.*, 1987

N/a – not applicable
NP-HPLC – normal phase HPLC
HRGC – high resolution GC
NP – nonylphenol
Fluor - fluorescence

Table 1.3 gives an overview of chemical methods of analysis though many others have been used, including supercritical fluid chromatography and capillary electrophoresis, these are reviewed by Lee, 1999. Methods of analysis for the APEs were initially performed using bismuth active substances (BiAS) [Waters *et al.*, 1986] and cobalt thiocyanate active substances (CTAS) [Thiele *et al.*, 1997]. They form donor-acceptor complexes with the non-ionic surfactants and may then be determined by colorimetry or potentiometric titration, these methods were replaced as a result of the poor sensitivity in comparison to the methods shown in Table 1.3.

1.4 Estuary overview

If the results for the water, suspended solids and sediment are to be correctly interpreted then a general understanding of estuarine dynamics is essential. This section presents an overview of the chemistry of an estuary system including ionic strength and composition, and pH variations (Table 1.4).

Table 1.4 *Comparison of river and estuarine composition.*

	Approximate total dissolved solids (mg/l)	Ionic composition	pH
River water	100	Ca^{2+}, Mg^{2+}, HCO_3^-, $H_4Si\,O_4$	5 - 9
Sea water	34 000	Na^+, Cl^-	8 (buffered system)

The estuary is the mixing zone between sea and river, the extent of this mixing is dependent on a number of factors and these will be discussed below.

1.4.1 Salinity

Seawater is of greater density than the freshwater of the river, therefore distinctive gradients in water salinity often occur as a result of estuarine water circulation, this is dependent on the flow of the river and the tidal range of the sea. There are 3 main salinity profiles, (Fig. 1.8) though any given estuary may not have a specific mixing type but may vary in terms of season as well as factors such as wind strength.

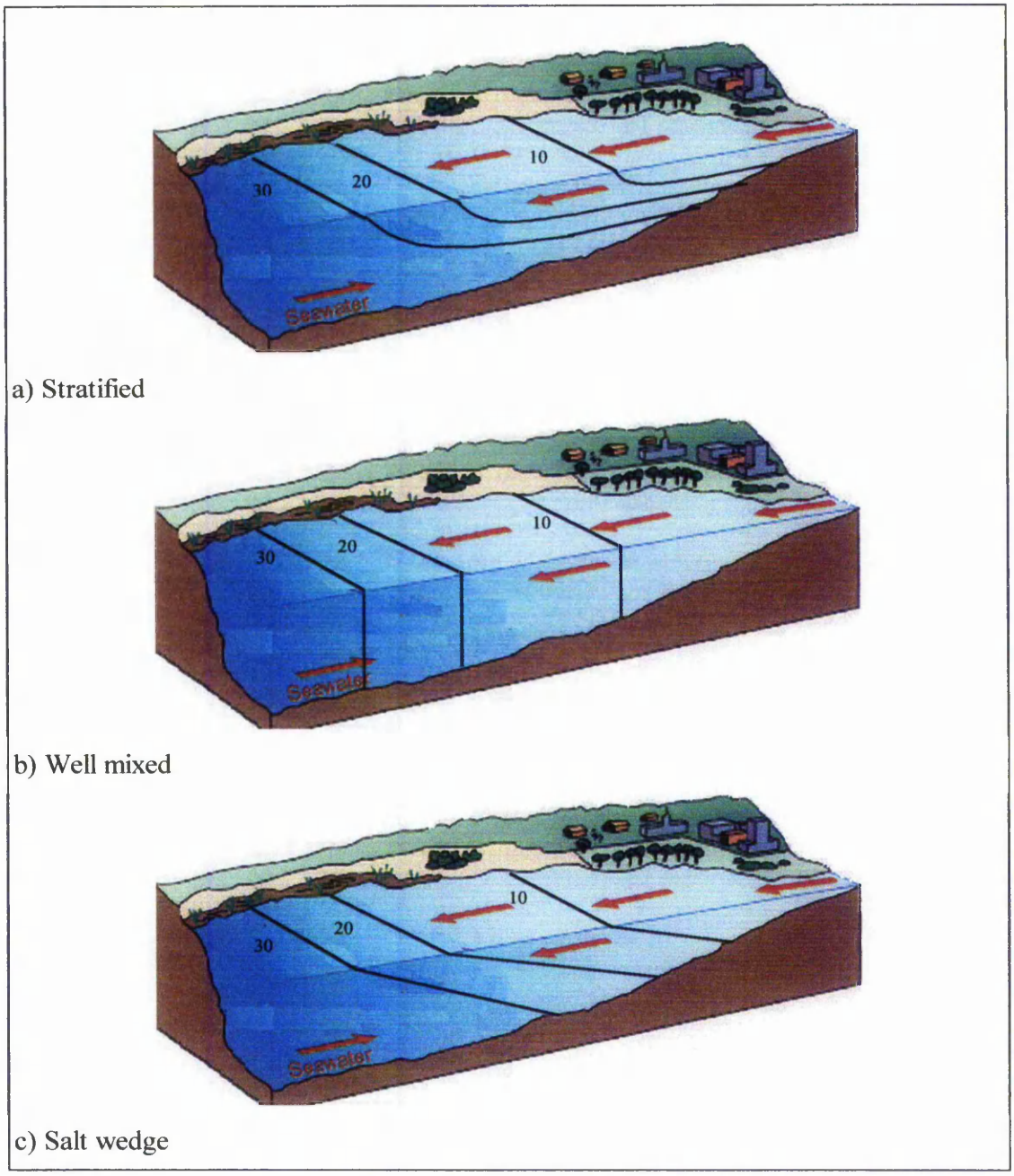

Figure 1.8 *Estuary salinity profiles, the figures correspond to the salinity g / l [Ross, 1995]*

1.4.2 Particulate matter

Estuaries receive particulate matter from both river and marine sources leading to problems of silting, often resulting in the need for dredging if the estuary is to be used by large vessels. River water also contains colloidal material barely distinguishable from dissolved matter, the flocculation of which is not possible as a result of electrostatic repulsion. However, with the change in ionic character upon entry to the estuary the colloids aggregate to form particulate matter that will then be subject to gravitational settling. The process is potentially enhanced by the change in pH. The movement of this particulate matter is now dependent on tidal flow as shown in Fig. 1.9.

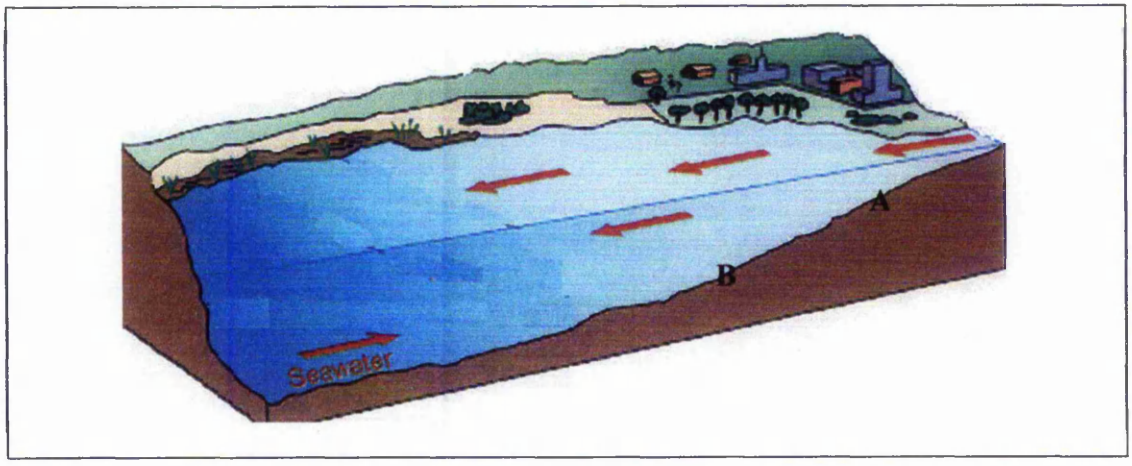

Figure 1.9 *Movement of particulate matter within an estuary system, points A and B indicate the main area within which particulate movement will occur [Ross, 1995].*

With an ebb tide of low speed and longer timescale, more particles will settle out of water with deposition occurring between A and B. However, with the returning flood tide of greater speed and shorter time (in comparison to the ebb) the settled particulates will become resuspended and transported back upstream to be redeposited at point A. A turbidity maximum zone (between points A and B) is often found in the upper part of the estuary and this is often accompanied by a decrease in dissolved oxygen, as a result of the high oxygen demand of organic carbon within the particulate matter.

1.4.3 Contamination of an estuary

Organic pollution within an estuary is a common problem. Estuaries are usually the site of much industrial activity and as a consequence also the site of a certain amount of industrial discharge, the river flowing into the estuary will also bring pollutants from agricultural areas and sewage effluents. These pollutants may not cause a problem in the river system, as they are associated with the suspended solids, upon entry to the estuary however, they are likely to flocculate and become incorporated into the sediment.

There are a number of factors that contribute to the persistence of a compound in an estuarine system, these include, water solubility, degradation, complex formation and adsorption to soils and sediments. These factors are in turn dependent on the water composition i.e., organic and salt content as well as pH and temperature.

1.5 Aims of this project

The main aim of this study will be to improve on existing extraction and analytical methods for the identification of alkylphenols, namely, nonylphenol and octylphenol, in a variety of environmental matrices. An analytical method will be developed to achieve maximum separation in the minimum analysis time, detection will utilise the fluorescent nature of the compounds of interest. A number of extraction methods will be determined and the principles will remain the same irrespective of matrix. Solvent volumes will be kept to a minimum, with the minimum sample volume used for maximum concentration. The extraction time will also be kept as short as possible. With all methods optimised, they will then be applied to environmental samples collected from the Forth Estuary.

The Forth Estuary was chosen for a number of reasons:

- This estuary has not been analysed for alkylphenols and would therefore produce valuable data in terms of building up a picture of alkylphenol discharges in UK water systems.
- There has been evidence of intersex in benthic copepods [Moore and Stevenson, 1994] in this estuary and it would be interesting to determine if alkylphenols may be a contributing factor to such an effect.
- There was also collaboration with SEPA (Scottish Environment Protection Agency) on this work, which allowed use of research vessels and facilitated sampling.

The proceeding chapters will endeavour to explore these aims in terms of the method development, and present the results of the environmental sampling. Conclusions will be drawn and comparisons made between the different matrices and the alkylphenol distribution within them.

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Chapter 2: Method Development

1.1 Introduction

This chapter will endeavour to explain the theoretical aspects of the techniques to be employed in this study. Analytical methods will include chromatography, solid phase extraction and fluorescence. Once methods have been established these techniques will be applied to the various environmental matrices discussed in the present study.

2.1.1 Chromatography

The first published data on the separation technique that later became known as chromatography was in 1903 by Tswett. He described the use of a glass column packed with a solid adsorbent to separate chlorophyll extracts using a liquid mobile phase [Lough and Wainer,1995]. There was little interest in this technique until 1930 when Lederer and co-workers used column liquid chromatography to separate the pigments in egg yolk [Kuhn and Lederer, 1931]. From this point, progress in chromatographic techniques increased rapidly with the development of thin layer chromatography (TLC), gas chromatography (GC), supercritical fluid chromatography and high performance liquid chromatography (HPLC).

2.1.2 High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) was established in the late 1960's and has evolved as a valuable analytical technique [Braithwaite and Smith, 1996]. The technique is often favoured over GC as there are no volatility or thermal stability constraints. The non-destructive nature of many of the detection systems, for example, UV, allows further analysis by other techniques such as mass spectrometry (MS) or infra-red spectroscopy (IR).

2.1.2.1 Instrumentation

The HPLC system (Fig. 2.1) is made up of a number of different components, outlined below.

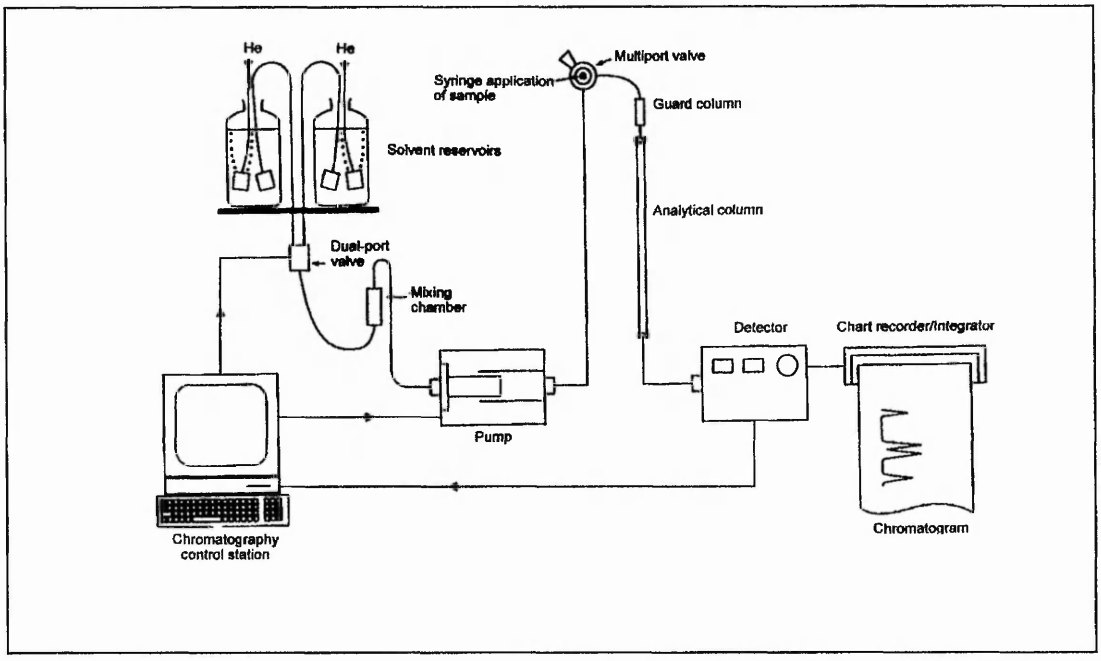


Figure 2.1 *Schematic of an HPLC system. [Braithwaite and Smith, 1996]*

The mobile phase solvents are filtered to remove particulate matter, degassed to remove bubbles and are of a high spectroscopic grade (the solvent has a specific UV wavelength cutoff). The solvents may be used individually, pre-mixed if an isocratic (non-gradient) mobile phase is employed, or mixed in a mixing chamber if gradient elution is required. With gradient elution, the mobile phase composition is altered during the run to improve resolution and analysis time. Disadvantages of this type of analysis include, a wandering baseline on the resulting chromatogram and increasing analysis time as a result of column re-equilibrated before each new run.

The mobile phase is pumped through the system using a pump that is capable of non-pulsed flow, providing a constant inlet or outlet pressure. The pumps are designed to withstand corrosion from the commonly used HPLC solvents and pressures up to 7000 psi (pounds per square inch).

The sample is introduced to the system by a 6 port injection valve. This allows precise sample loading onto the column with minimum disruption to the flow of the mobile phase. Once injected the sample is swept onto the HPLC column by the flow of the mobile phase.

The column is usually between 10-25 cm long and 2.1 - 4.6 mm in internal diameter with construction of stainless steel to withstand the high back pressures generated. The stationary phase is usually composed of uniform silica particles (3-10 μm) with surface bonded groups such as C_{18} or NH_2 attached [Braithwaite and Smith, 1996] to improve analyte separation (this is discussed further in Section 2.1.3.2).

Once separated the analytes of interest are carried into the detector. The choice of detector is dependent on the specific properties of the analytes, for example, UV absorbance is possibly the most versatile system as a wide range of analytes can be detected. However, if the analytes of interest are of a certain compound class and could be detected by a specific property such as fluorescence, then selectivity would be increased as interferences would be unlikely to possess the same spectroscopic properties and would therefore not be in a position to interfere (see Section 2.1.4).

2.1.2.2 HPLC theory

Chromatography is a technique used to separate a multi-component mixture using a stationary phase and mobile phase. For HPLC the stationary phase will be a solid support with a liquid mobile phase. The multi-component mixture is separated by the interaction of each component with the stationary phase and the resulting distribution between the stationary and mobile phase. Each component will have relative affinity for the stationary phase and will therefore be eluted through the column at different retention times (Fig. 2.2).

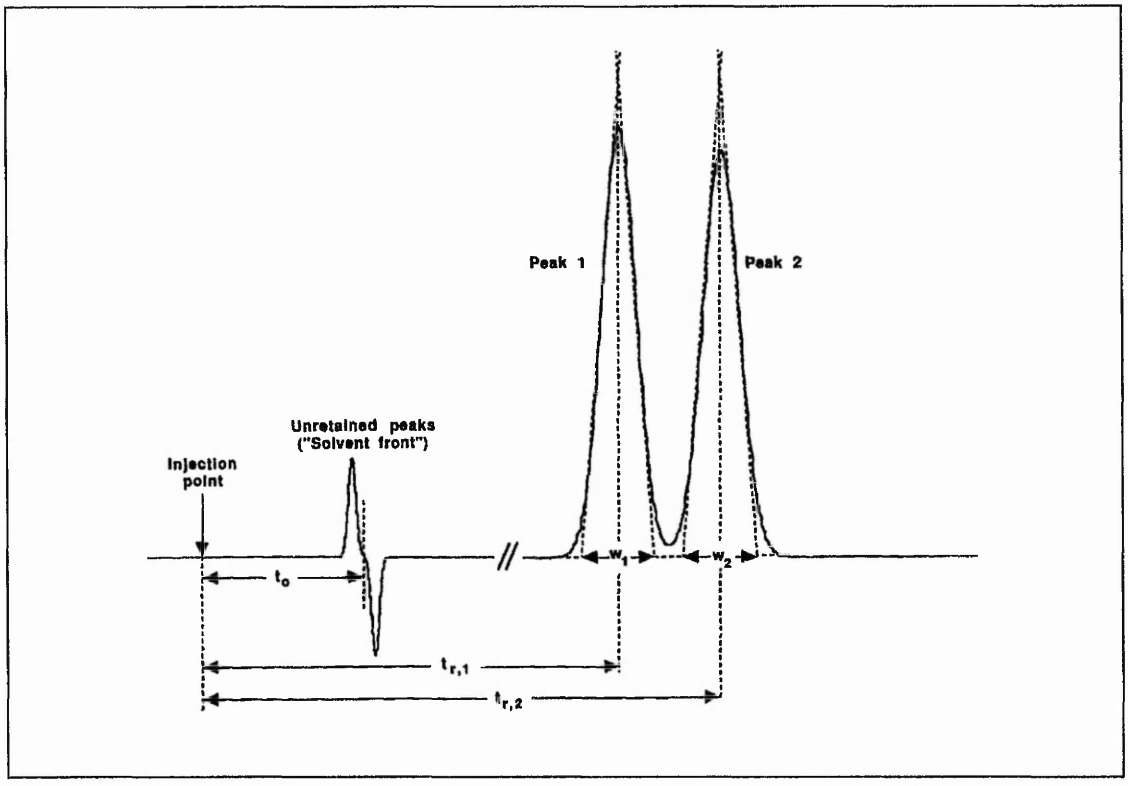


Figure 2.2 *Example chromatogram with retention time (t_r) and peak width (W) marked [Lough and Wainer, 1995].*

There are a number of important parameters in chromatography that need to be introduced, the definitions and equations below are those most commonly used [Lough and Wainer, 1995, Braithwaite and Smith, 1996].

Distribution ratio (capacity factor, partition coefficient)

Each analyte has a retention characteristic dependent upon its relative affinity for both the mobile and stationary phases. This affinity is expressed as the distribution ratio, K.

$$K = \frac{C_s}{C_m} \qquad \textbf{Equation 2.1}$$

Where:

C_s= concentration of analyte in the stationary phase.

C_m= concentration of analyte in the mobile phase.

The larger the value of K, the greater the affinity for the stationary phase, therefore the longer it takes for the analyte to elute from the column.

Column efficiency

The column efficiency is defined by the number of theoretical plates (N) in a column (Eq. 2.2). These plates may be imagined as hypothetical layers in a column with the analyte in a state of equilibrium between the mobile and stationary phases.

$$N = 16\left(\frac{t_r}{W_b}\right)^2 \qquad \textbf{Equation 2.2}$$

Where:

t_r = retention time.

W_b = peak width at base.

The number of theoretical plates are directly proportional to the column length (L) and inversely proportional to the particle size (d_p). Therefore with an increase in column length there is an increase in the number of theoretical plates but as particle size increases the number of plates decrease (see following section).

Band Broadening

There are a number of factors that can affect band broadening (i.e spread of analyte on elution from the column) and they can be divided into two main groups, non-column and column effects. Non-column band broadening occurs as the analyte encounters dead volume within the chromatographic system. These effects can be kept to a minimum by using well-designed instrumentation and fittings that will reduce dead volume and improve resolution. Column band broadening is summarised by the van Deemter equation (Eq. 2.3) and results from three processes, namely eddy diffusion, longitudinal diffusion and resistance to mass transfer.

$$H = A + \frac{B}{\nu} + C_s\nu + C_m\nu \qquad \textbf{Equation 2.3}$$

Where:

H = height equivalent to a theoretical plate $= \frac{L}{N}$

A = eddy diffusion.

B = longitudinal diffusion.

C_s = resistance to mass transfer in the stationary phase.

C_m = resistance to mass transfer in the mobile phase.

ν = average linear velocity. $= \frac{L}{t_o(dead\ time)}$

Eddy diffusion occurs as the analyte moves randomly through the particles of the stationary phase, therefore travelling at different distances over a set length of column. The stationary phase particles may also cause eddys in the mobile phase resulting in mixing and dispersion of molecules (Fig. 2.3). To achieve good separation, small diameter particles (d_p) should be used with uniform packing in the column (Eq. 2.4), where λ is a constant that takes into account packing uniformity and column dimensions.

$$A = \lambda d_p \qquad \textbf{Equation 2.4}$$

Longitudinal diffusion (Eq. 2.5) takes into account the random motion of the analyte molecules, diffusion is independent of flow direction and takes place along the axis of the column. This form of diffusion is dependent on time, the longer the analyte band takes to pass through the column the more time available for diffusion to take place.

$$B = 2\gamma D_M \qquad \textbf{Equation 2.5}$$

Where:

γ = hindrance factor.

D_M = diffusion coefficient of component in mobile phase.

The resistance to mass transfer refers to the interactions of the analyte to the stationary phase and is shown in Fig. 2.3. This is an important factor in band broadening for both GC and HPLC with mass transfer of both mobile (C_m) and stationary phase (C_s) being of significance.

$$C_s = \frac{d_f^2}{D_S} \qquad C_m = \frac{d_p^2}{D_M} \qquad \textbf{Equation 2.6}$$

Where:

d_f = stationary phase film thickness.

D_S = diffusion coefficient of component in stationary phase.

D_M = diffusion coefficient of component in mobile phase.

d_p = diameter of stationary phase particles.

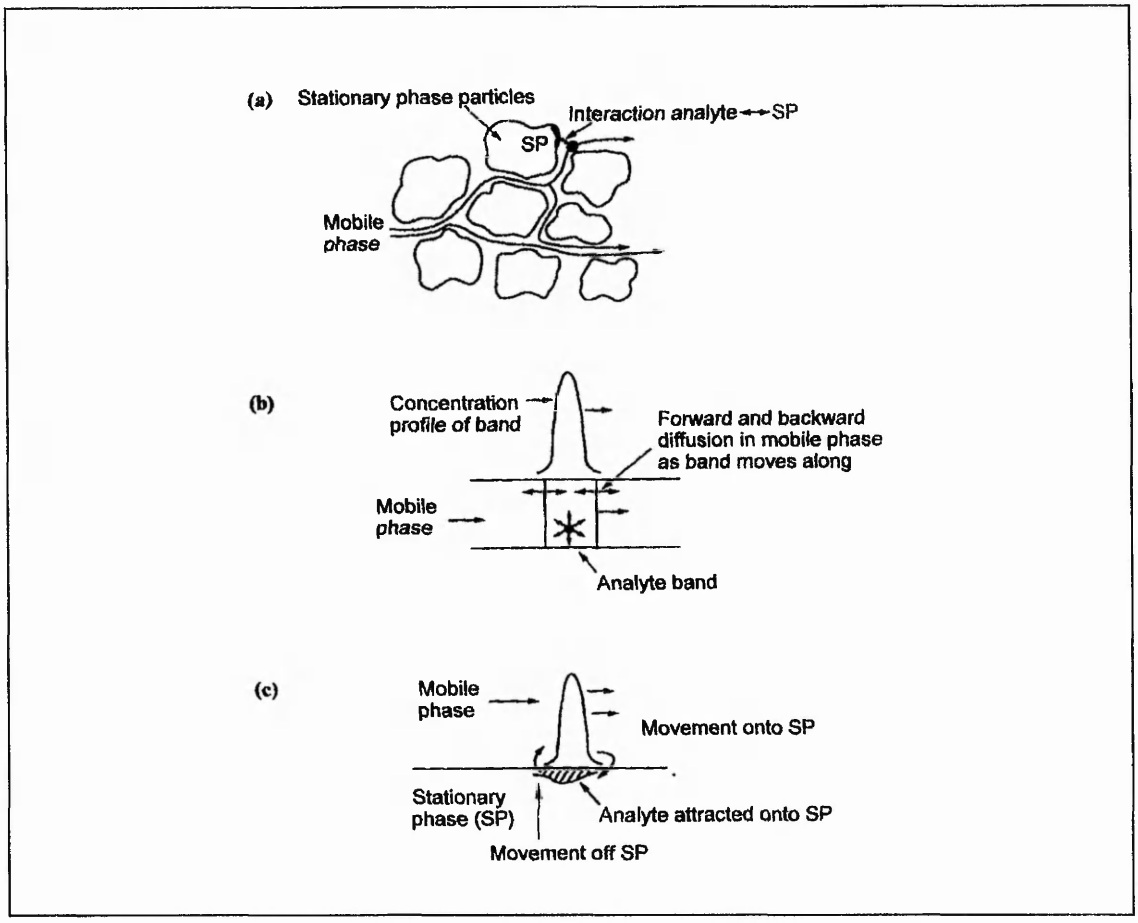


Figure 2.3 *van Deemter diagrams, a) eddy diffusion, b) longitudinal diffusion and c) resistance to mass transfer [Braithwaite and Smith,* 1996].

Selectivity

Selectivity is the term used for the retention ability of the stationary phase for a variety of analytes. The selectivity (α) for adjacent peaks is expressed in terms of the retention times of two solutes, Eq. 2.7, the definitions are shown in Fig. 2.2.

$$\alpha = \frac{t_{r2} - t_0}{t_{r1} - t_0} \qquad \textbf{Equation 2.7}$$

This equation does not take into account band broadening and the effect of peak width, therefore the stationary phase may be selective for the analytes but as a consequence of other column effects the peaks may not be resolved.

Resolution

The ability of a chromatography column to separate two adjacent peaks is termed resolution. Peak resolution is dependent upon a number of factors, retention characteristics of each analyte (k), the selectivity of the stationary phase (α) and the column efficiency (N). Resolution (R_s) is calculated using Eq. 2.8

$$R_s = \frac{t_{r2} - t_{r1}}{0.5\,(W_2 + W_1)} \qquad \textbf{Equation 2.8}$$

Resolution should ideally give a value of between 1.2 and 1.5 [Braithwaite and Smith, 1996] where the corresponding peak overlaps by 1% and 0.2%. There is a fine balance between excellent resolution and analysis time, if R_s is greater than 1.8 then the chromatographic method should be altered to reduce analysis time and decrease band broadening of the later eluting peaks.

This concludes the HPLC section in terms of theory with an application of some of the equations shown in Section 3.2 and 4.2. The following section looks at solid phase extraction and links in well with the discussion on chromatography as it is based on the same principles, though on a more rudimentary level. The HPLC detection system will be discussed in Section 2.1.4.

2.1.3 Solid Phase Extraction

Solid phase extraction (SPE) is a preparative technique with a wide range of applications, including, sample cleanup, pre-concentration and solvent exchange. The theory behind the technique is closely linked to that of chromatography and offers a number of advantages over traditional extraction methods:

- small solvent volumes are required.
- highly reproducible recoveries are achievable.
- there are no expensive glassware requirements.
- the SPE system is easily automated.

Another advantage is the compatibility of the extraction method with both GC and HPLC analysis.

2.1.3.1 Principles of SPE

The basic principles of SPE are chromatographically based and are illustrated in Fig. 2.4. The sample is passed through the SPE cartridge and the compounds are absorbed into the packing material of the cartridge. Unwanted analytes or interferences are removed by 'washing' the cartridge with a suitable solvent, with the elution solvent selectively removing the analyte compounds. The extraction is usually carried out under vacuum, although alternatively positive pressure or centrifugation may be employed.

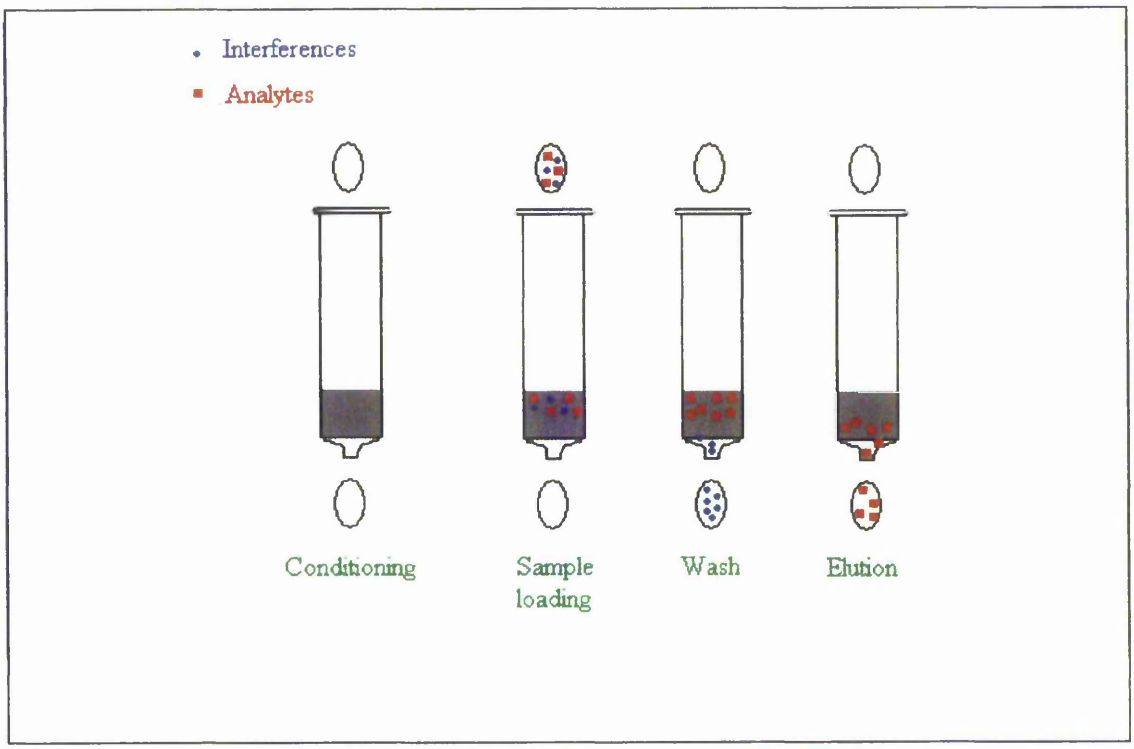


Figure 2.4 *Principles of solid phase extraction.*

The packing material is usually of a silica base as it is highly porous. Careful control during manufacture ensures uniform, reproducible properties. The silica may be used directly as a packing sorbent or functional groups may be attached to the silica surface to achieve a range of sorbents with various extraction properties. The basic silica backbone is shown in Fig. 2.5 with the C_2 functional group attached, this group can be replaced by any of the groups displayed in Table 2.1.

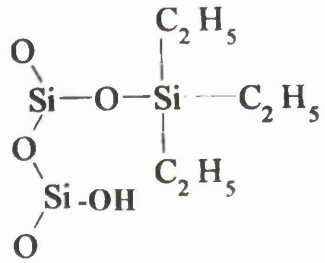


Figure 2.5 *SPE silica backbone with attached C_2 functional group.*

The modified silica packing materials will still retain a number of active silanol sites (Fig. 2.5), these sites can be responsible for secondary polar and ionic interactions. The presence of these sites in many applications are beneficial and can be used to improve the extraction process. However, these silanol groups can cause unwanted interactions and can therefore be deactivated by reacting with trimethyl silane (a term known as endcapping). There are a wide range of sorbents available and these can be grouped into three main headings, polar, non-polar and ion exchange. Examples are shown in Table 2.1.

Table 2.1 *Examples of SPE packing sorbents.*

Type of packing	**Non-polar**	**Polar**	**Ion exchange**
Matrix:	Aqueous	Non-polar	Aqueous
Packing sorbent	C_{18-1} PH (phenyl) CN (cyano)	CN Silica Diol NH_2 PSA (ethylenediamine -N-propyl)	NH_2 PSA SAX (quaternary amine) CBA (carboxypropyl) SCX (benzenesulphonic acid) PRS (propylsulphonic acid)

2.1.3.2 Selection criteria

When choosing the sorbent material for the SPE packing it is important to define the necessary requirements of the packing material. Batch screening is an efficient way to assess analyte retention and in turn the most suitable sorbent. Once the appropriate sorbent has been determined the method is optimised to achieve high recoveries with good reproducibility and removal of interferences, this is achieved by altering solvent polarity and volume.

2.1.4 Detection system

The HPLC detector has a number of requirements including, good selectivity and sensitivity, low noise, sufficient linear range and precision. There is also a need for dynamic analysis as the HPLC system is flowing constantly. Table 2.2 shows a list of common detectors and their properties. Of particular interest in this current work is fluorescence detection (Section 2.1.4.1).

Table 2.2 *HPLC detectors and their properties [Snyder and Kirkland, 1979].*

	Type	Sensitivity to favourable sample	Gradient elution	Flow sensitive
UV-visible	selective	2×10^{-10} g / ml	Yes	No
Fluorescence	selective	10^{-11} g / ml	Yes	No
Refractive index	general	1×10^{-7} g / ml	No	No
Electrochemical	selective	10^{-12} g / ml	No	Yes

2.1.4.1 Fluorescence

Fluorescence occurs when light is absorbed by a compound in its ground electronic state causing an electron to be promoted to a higher electronic state. Some of this energy is then lost *via* internal conversion and vibrational relaxation until the lowest vibrational level of the excited electronic state is reached. At this point a number of possibilities arise:

- The electron may return to the ground state by emission of a photon, this is known as fluorescence.
- The electron may return to the ground state by internal conversion (IC), (a non-radiative transfer).
- The electron may also undergo intersystem crossing (ISC) from a singlet to a triplet state, this is again non-radiative, though the transfer of an electron to the ground state from the triplet results in radiative phosphorescence.

The Jablonski diagram in Fig. 2.6 illustrates the absorption and emission of light as described above.

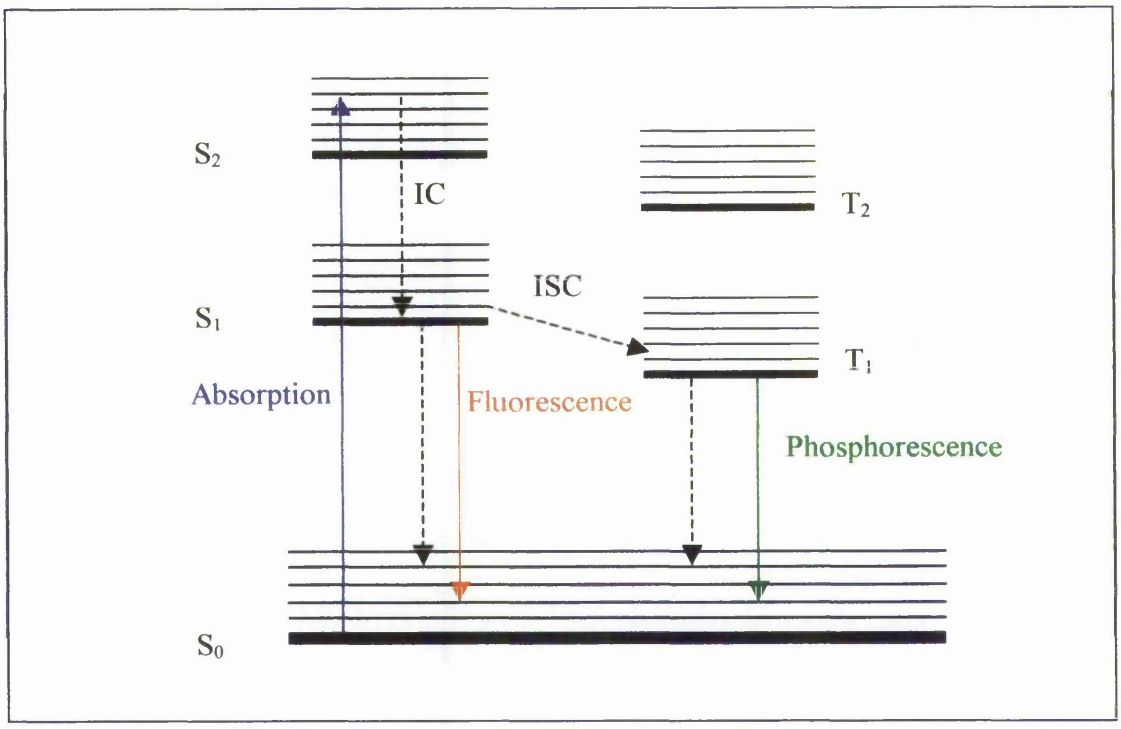


Figure 2.6 *Jablonski diagram of luminescence pathways; the thick lines indicate ground vibrational levels and the thin lines denote higher vibrational levels. Singlet (S) and triplet (T) states*

A number of factors can influence the fluorescence intensity. These include temperature, pH and viscosity of the mobile phase, as well as quenching caused by the presence of solutes. The reduction in energy from absorption to fluorescence emission results in an increase in the wavelength from the excitation to emission.

The use of fluorescence as a detection technique has increased significantly over recent years as a result of improvements in the HPLC system and alterations to the detection system to allow for flow through analysis. Fluorescence offers good selectivity, specificity and sensitivity. The selectivity of this method is possible as fewer compounds possess the ability to fluoresce, therefore exhibiting greater selectivity in comparison to UV detection. Specificity is achieved as compound classes have defined emission and excitation spectra therefore removing the detection of interference (unless these are of the same structure). The sensitivity of fluorescence is possible as the background signal is ideally zero and the only signal detected would be the result of a fluorescent compound in the flow cell.

2.1.4.2 Instrumentation

The basic fluorimeter and its components are shown in Fig. 2.7.

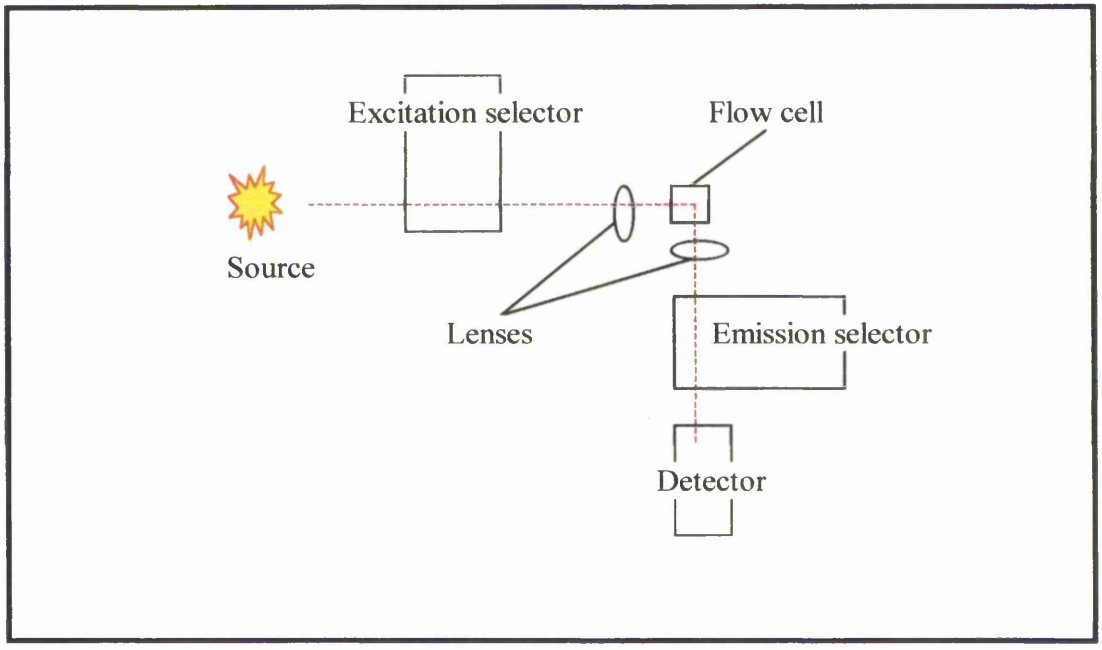


Figure 2.7 *Schematic diagram of a fluorescence detector.*

The source is usually a xenon or deuterium arc lamp as they deliver a broad spectrum extending into the UV region. The excitation wavelength selector can be a filter or a monochromator, each having their own advantages. The filter gives greater throughput though the monochromator has greater versatility with the ability to scan as well as providing better resolution. The flow cell is made of quartz to allow analysis in both the UV and visible regions, with dimensions kept to a minimum to prevent loss of resolution. The emission selector is again a filter or monochromator as for the excitation selector, though set at 90° to the excitation to reduce scattered light interferences. The detector set at the exit from the emission selector is a photomultiplier tube.

There are 2 main groups of compounds that can be detected using fluorescence, those that fluoresce naturally and those that can be induced to fluoresce by derivatisation. The naturally fluorescent compounds usually possess an extensive π electron system, these are often found in aromatic and highly conjugated systems, for example, PAHs, pesticides, steroids and vitamins [Vickrey, 1983]. Only 5-10 % of all compounds have the ability to fluoresce naturally, of the small percentage that do fluoresce there are few that will have the same wavelengths of excitation and emission.

2.2 Analytical Method Development

In this work three main analytical techniques have been used for the analysis of alkylphenols, these are GC - MS, reverse phase HPLC and normal phase HPLC. These methods have been discussed in Chapter 1 and will be considered in a practical sense in this section.

2.2.1 Reagents

Technical grade nonlyphenol (NP), 4 –tert.-butylphenol (BP), 4 –n- octylphenol (OP) and anhydrous sodium sulphate were supplied by Aldrich (Poole, UK), sodium chloride was supplied by Fisher (Loughborough, UK). The solvents were all HPLC grade and supplied by Rathburn (Peeblesshire, UK).

2.2.2 GC - MS

GC - MS was examined as a possible technique for the analysis of the alkylphenols as both quantitative and qualitative analysis could be employed. The GC - MS used was a Hewlett Packard 5890 series II GC coupled to a Hewlett Packard 5971 quadrupole mass selective detector system. The capillary column was a BP5 20 m long, 0.32 mm diameter with a 0.25 μm film thickness. The temperature programme used was:
40°C for 50 seconds, 20°C / minute to 325°C and held for 30 minutes.

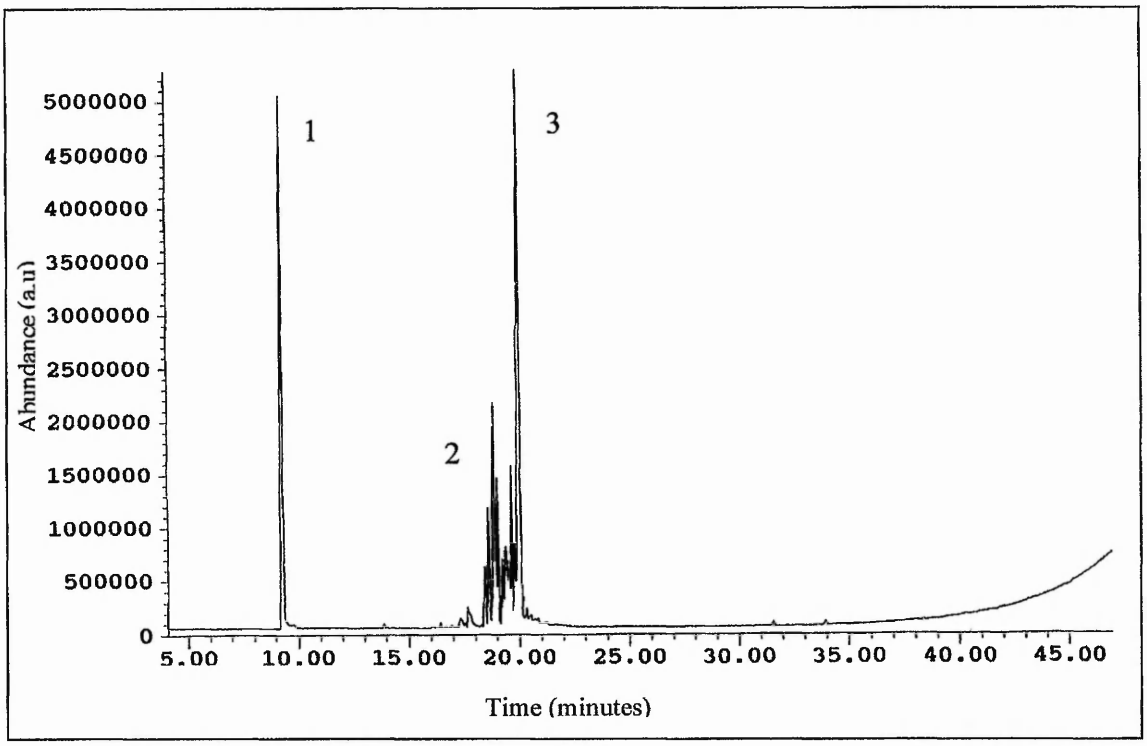


Figure 2.8 *GC - MS spectra of analytes 1) Butylphenol, 2) Nonylphenol and 3) Octylphenol*

The results were disappointing in comparison to the literature as the lowest concentration that could be detected was 100 mg / l (Fig. 2.8). The chromatogram also illustrated another problem with the GC method as nonylphenol was present as a number of isomeric peaks, therefore making quantification difficult. The GC – MS system used for this experiment was not optimised for the detection of alkylphenols but was a general use system, this may explain the difference in these results with those found in the literature. The GC - MS method was concluded to be unsuitable for the analysis of alkylphenols as a consequence of the high limit of detection and the poor quantification of the nonylphenol peak.

2.2.3 HPLC

HPLC was investigated as a potential analytical method for the separation of APs, with the results of both reverse and normal phase chromatography discussed, as well as examining the practical aspects of fluorescence detection.

2.2.3.1 Detector

Fluorescence as a means of detection is cited in the literature [Marcomini *et al.*, 1990, Holt *et al.*, 1986]. The optimum wavelengths of excitation and emission were determined on a scanning fluorimeter, the Perkin Elmer luminescence spectrometer LS 30. The excitation was set at 230 nm and the emission was scanned, with the maxima indicating the optimum emission wavelength. Fig. 2.9a indicates the maxima at 306 nm, the excitation was then scanned using the determined emission wavelength Fig. 2.9b. The excitation maxima was 220 nm, at this wavelength a number of the solvents are known to interfere, therefore 230 nm was chosen and the problem of interference was reduced. These wavelengths are similar, if not the same as those used in the literature [Holt *et al*, 1986, Marcomini *et al.*, 1990, Ahel and Giger, 1993].

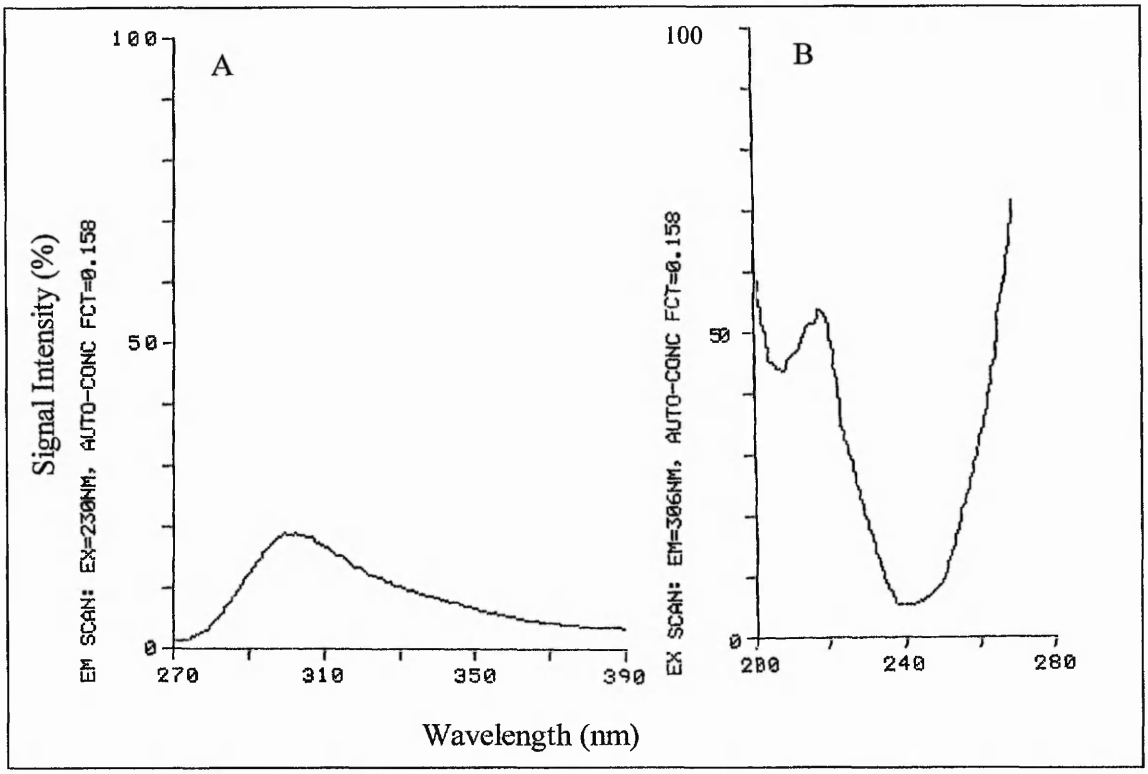


Figure 2.9 *Scanning a nonylphenol standard for optimum wavelengths of A) excitation with emission set at 230 nm and B) emission with the excitation set at 306 nm (the large peak at 270nm is the scatter peak).*

2.2.3.2 Reverse phase HPLC

Reverse-phase HPLC uses a non-polar stationary phase with a polar mobile phase. All reported reverse phase work for AP determination was applied to a C_{18} column following the method by GL Sciences, Japan. The mobile phase was 70 : 30 acetonitrile / 20 mM potassium phosphate buffer (pH 3), a flow rate of 1 ml / minutes and the UV wavelength set at 280 nm. Fig. 2.10 shows the resulting chromatogram for a 1 mg / l standard of the 3 analytes, nonylphenol, octylphenol and butylphenol.

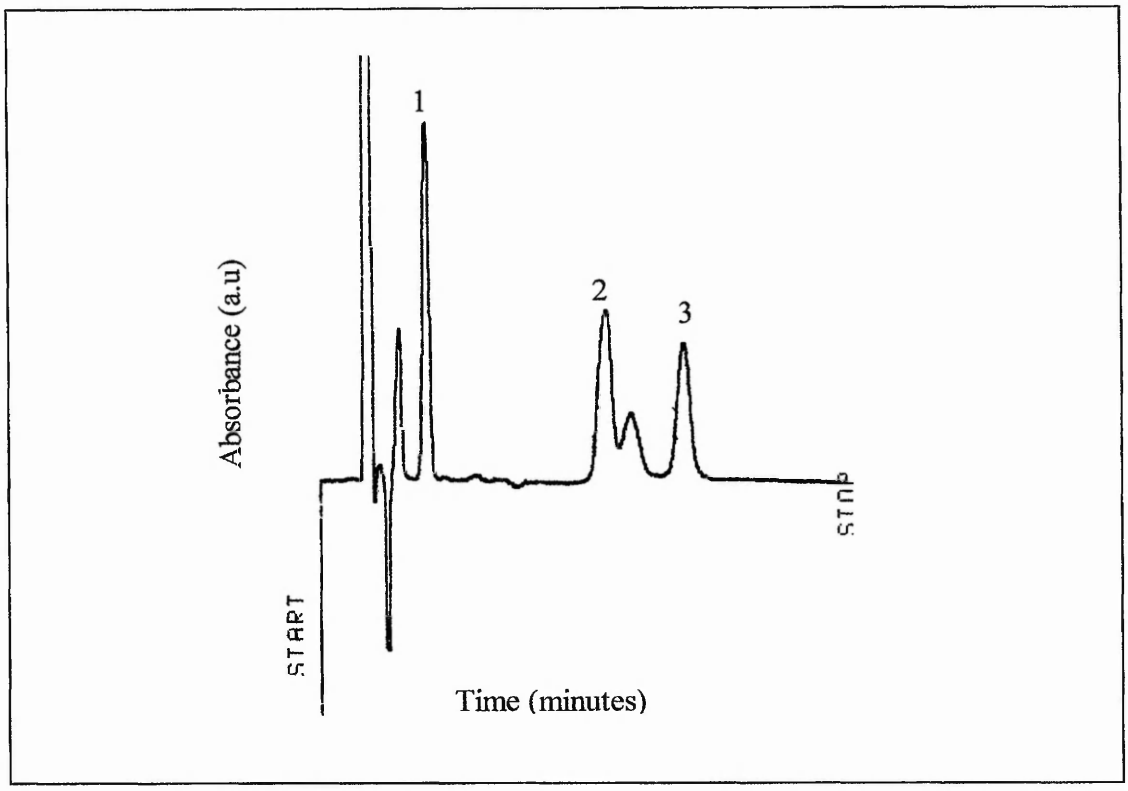


Figure 2.10 *Reverse phase HPLC chromatogram using UV detection of 280 nm. 1) Butylphenol, 2) nonylphenol and 3) octylphenol*

The range of detection was between 1 and 0.1 mg / l, an improvement compared to the GC - MS system but still not sensitive enough for the detection of the anticipated environmental concentrations (unless a large concentration step was employed). Fluorescence detection was evaluated, achievable detection limits were very similar to those obtained using UV. The reason for the lack of sensitivity using fluorescence detection could be the quenching effect of the water present in the mobile phase of this reverse-phase system, the problem is not encountered when using normal-phase as shown in the following section.

2.2.3.3 Normal phase HPLC

A normal phase system requires a polar packing material, the literature indicated aminopropyl to be most suitable for the separation of alkylphenols [Kvestak *et al.* 1994, Holt *et al.,* 1986]. The column dimensions were 250 × 4.6 mm with 5 μm particles, supplied either as an Apex column by Jones (Hengoed, UK) or as a Primesphere by Phenomenex (Macclesfield, UK). The main problem with the NH_2 packing is its vulnerability to carbonyl compounds. In certain circumstances they may react with the amino group to produce imines (Schiffs bases). The reaction is irreversible and may result in the column being rendered unusable or at least the peak elution times will increase markedly [Lough and Wainer, 1995].

There was no indication in this study that this type of degradation occurred when standards were analysed. However, when environmental samples were analysed the column needed to be replaced more often, this may have been a result of interference compounds reacting with the NH_2 groups or irreversibly binding to the sorbent under the conditions of the system. In some cases flushing the column with a range of varying polarity solvents could regenerate the column. The solvents used were:
Mobile phase - MTBE – methanol – water - methanol – MTBE – mobile phase.
A guard cartridge system was employed to reduce the impact of the environmental samples on the column and this would be assumed to increase the lifetime of the column.

In this current study a variety of mobile phases were experimented with including a gradient run of methyl tert.-butylether (MTBE) – acetonitrile/ methanol (95 : 5), MTBE (0.1 % acetic acid), MTBE / heptane (80 : 20) and MTBE / cyclohexane, for this solvent mix a number of combinations were employed. The combination found to be most suitable for the separation of the alkylphenols was 70 : 30 mix of cyclohexane / MTBE based on good resolution of OP and NP. The flow rate was set at 1.5 ml / minute on an isocratic mode to gain optimum separation and a run time of less than 20 minutes. The instrumentation used for this work was comprised of:

Pump - Perkin Elmer binary LC 250 ,

Autosampler - Perkin Elmer ISS 200 Advanced LC processor with a 50 µl injection loop.

Column chiller - Jones Chromatography, model 7955 (set at 20°C).

Fluorescence detector – Varian 9070 or a Perkin Elmer luminescence spectrometer LS 30.

An example chromatogram is shown in Fig. 2.11.

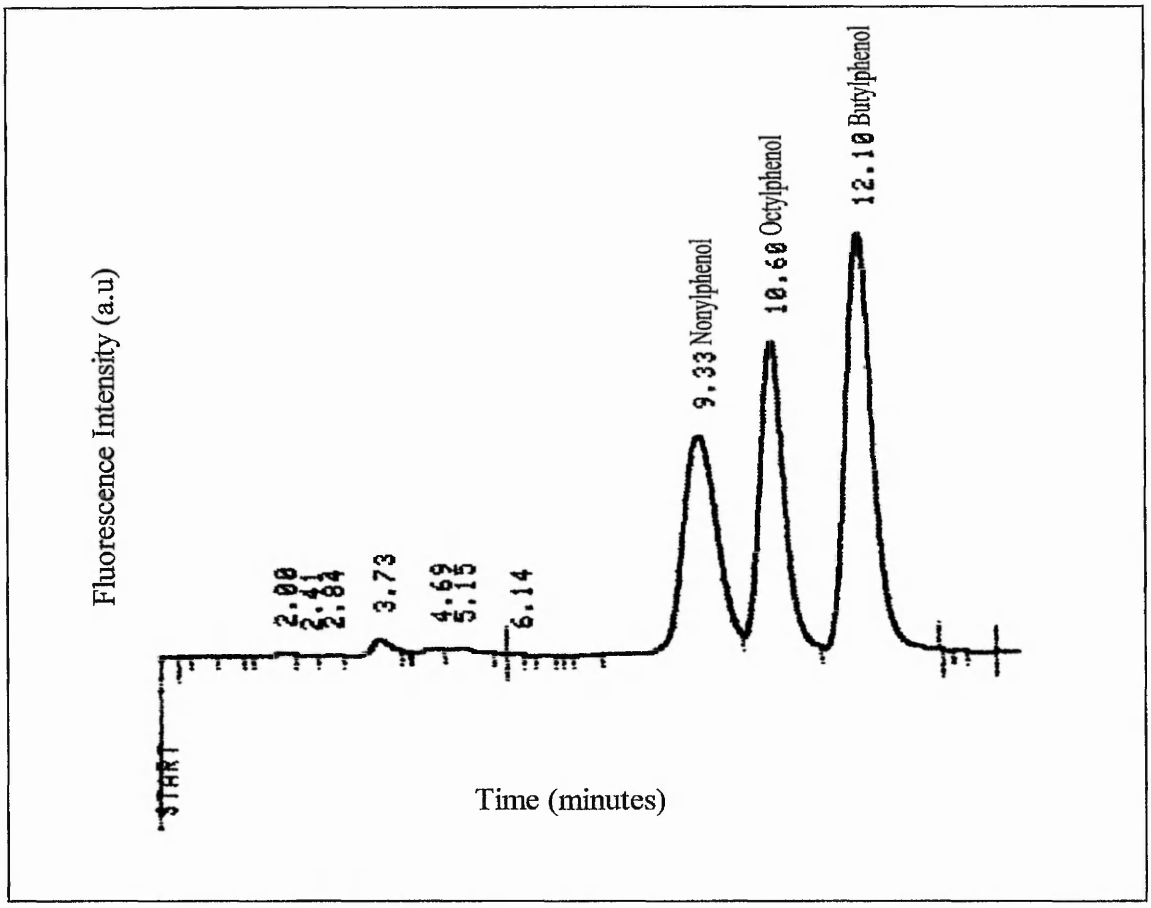


Figure 2.11 *An example chromatogram of a 1 mg / l standard in ethyl acetate of nonyl-, octyl- and butylphenol using normal phase HPLC.*

The calibration of the analytical method will be presented in Chapters 3 and 4 as different analysis solvents are used depending on application.

2.2.4 Calibration methods

There are a number of methods that can be employed for the quantification of analyte peaks, the two most common methods compare and calculate unknown concentrations by the application of an internal or external standard.

2.2.4.1 Internal calibration

An internal standard can be added prior to extraction to allow for extraction efficiency, or prior to injection to allow for variations that may occur within the analytical instrumentation. The internal standard (IS) is selected based on the following criteria:

- The internal standard should be of similar structure to the analytes of interest to give comparable extraction efficiencies.
- The internal standard must also appear in a blank region of the chromatogram though close to the analytes of interest.
- It should be stable, therefore, unreactive with the analytes of interest, column packing or mobile phase.

When using an internal standard calibration method a standard chromatogram containing all compounds for analysis and the internal standard is produced. From this chromatogram the detector response factor (Drf) is calculated for each individual analyte using Eq. 2.9.

$$Drf = \frac{Area\ of\ analyte}{Area\ of\ IS} \times \frac{Concentration\ of\ IS}{Concentration\ of\ analyte} \qquad \textbf{Equation 2.9}$$

Using the Drf value it is possible to calculate the unknown concentration of the particular analyte by applying Eq. 2.10 to a chromatogram of unknown analyte concentration.

$$Unknown\ concentration = \frac{Area\ of\ analyte}{Area\ of\ IS} \times \frac{Concentration\ of\ IS}{Drf} \qquad \textbf{Equation 2.10}$$

2.2.4.2 External calibration method

There can be difficulty in finding a suitable IS and with greater automation of the HPLC system there is a reduction in instrumental variation. With this in mind an external calibration can be used by running a standard solution every three or four samples. From this standard chromatogram a response factor (Rf) can be calculated (Eq. 2.11) using the concentration and the peak area of the compound of interest.

$$\text{Response factor} = \frac{Concentration}{Area} \qquad \textbf{Equation 2.11}$$

The response factor is then applied to a chromatogram of unknown concentration using Eq. 2.12.

$$Unknown\ concentration\ of\ analyte = Area\ of\ analyte \times Rf \qquad \textbf{Equation 2.12}$$

The two methods were compared using data generated from a standard calibration run and displayed in Fig. 2.12.

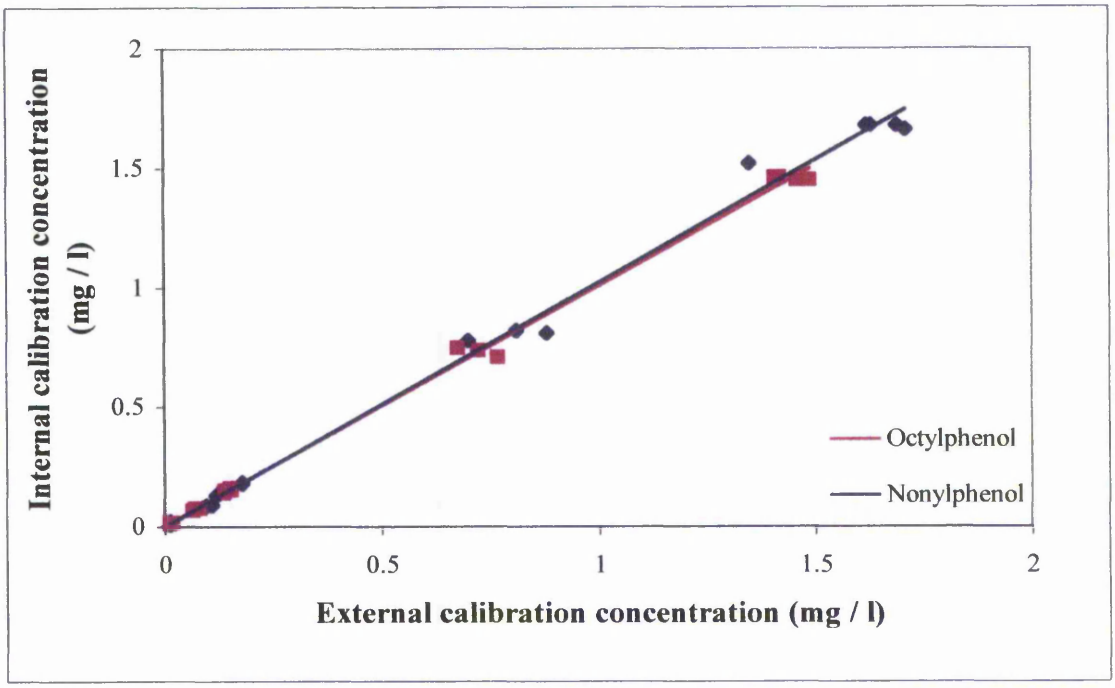


Figure 2.12 *A scatter plot of identical results calculated using an internal and an external calibration method.*

Both nonyl- and octylphenol results appear to give a 1:1 ratio indicating little difference between the calibration methods. This would suggest that should problems occur with the internal calibration, for example, interference problems or inconsistent extraction then the external method can be adopted with little or no effect on the calculated concentration measured.

2.3 Alkylphenol extraction from an aqueous matrix.

Direct analysis of alkyphenols from an aqueous matrix is problematic as a result of a number of factors:

- The anticipated low ppb level of the alkylphenols will require pre-concentration in order to be within the limits of detection.
- Detrimental effects of water on the normal-phase HPLC column and the detection system. The water needs to be replaced by a more compatible solvent.

Two methods of extraction and pre-concentration were investigated and both approaches are discussed in Sections 2.3.1. and 2.3.2.

2.3.1 Liquid- liquid extraction

Initially, liquid-liquid extraction by continuous extraction was evaluated using the apparatus shown in Fig. 2.13.

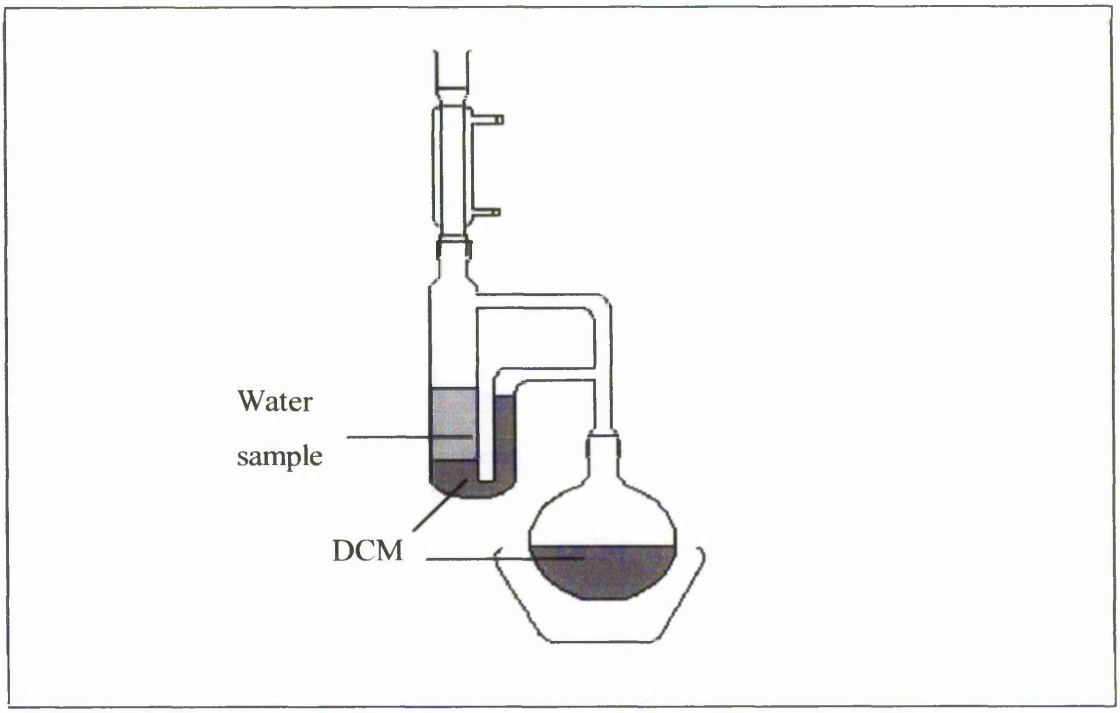


Figure 2.13 *Continuous liquid-liquid extraction apparatus.*

75 ml of water sample spiked to a known concentration with nonylphenol and butylphenol was extracted into 150 ml of dichloromethane (DCM) by refluxing for 2 hours. DCM was the solvent of choice as it was both immiscible with and of greater density than water. Both phases were transferred to a separating funnel where the aqueous layer was removed. Passing it through a filter containing anhydrous sodium sulphate, into a round bottom flask, chemically dehydrating the DCM layer. The DCM was then removed using rotary evaporation and the residue redissolved in 10 ml methyl-*tert.*-butyl ether (MTBE) prior to analysis. The reason for using MTBE as the injection solvent was its compatibility with the HPLC system and the ease with which the contents of the flask were redissolved.

The extraction procedure gave recoveries in excess of 100 %. The results were, however, reproducible indicating a systematic bias rather than random error. These high recoveries were possibly caused by contamination from an undetermined source. Potential contamination from glassware and solvents was investigated, but none of these potential sources were clearly identified as the source of the problem. For this reason the extraction method was rejected for the analysis of the environmental samples.

The second type of liquid-liquid extraction to be considered was that of liquid-liquid shake extraction. A 75 ml water sample was taken and spiked as for the continuous extraction method, 10 g of sodium chloride added to prevent emulsion formation. The water sample was shaken vigorously with 3 × 50 ml portions of DCM, the solvent layer was then filtered through anhydrous sodium sulphate. The filtrate was evaporated to dryness and redissolved in 10 ml of MTBE. The results are summarised in Table 2.3.

Table 2.3 *Preliminary recovery (%) results for the shake extraction method using distilled and de-ionised water at a spiked concentration of 1 ppm.*

	Compound	Mean % recovery	RSD (n=3)
Distilled water	Nonylphenol	86	5
	Butylphenol	77	9
De-ionised water	Nonylphenol	86	5
	Butylphenol	107	4

The results were a significant improvement on the continuous liquid-liquid extraction technique, as the recoveries do not indicate that contamination is a serious problem. Shake extraction also exhibited reproducibility and the systematic error that was present in the continuous extraction technique is absent from this extraction method. However, this technique has the disadvantages of using large solvent volumes and employs lengthy extraction times, therefore, solid phase extraction was investigated.

2.3.2 Solid Phase Extraction (SPE)

A number of SPE packing materials were screened with varying degrees of success. The initial selection criteria for cartridge type was the compatibility with an aqueous sample matrix, this included all alkyl chain packings as well as graphite carbon black and a diol packing material.

A variety of packing materials were screened for suitability of use. The methods and results are summarised below (Fig. 2.14), the volumes of solvents used were based on manufacturers recommendations. The cartridges were supplied by Alltech unless otherwise stated, containing 500 mg of packing material and with a capacity of 3 ml. The water sample was spiked to a concentration of 1 mg / l with all three analytes.

C_{18}

Condition - 5 ml methanol, 5 ml de-ionised water
Sample - 75 ml spiked water
Wash - 5 ml methanol
Elution - 10 ml acetone

All fractions were collected and taken to dryness under rotary evaporation before taking up in MTBE for analysis. From the results a nonylphenol peak could be determined in all fractions though the majority was seen in the methanol wash with a recovery of 41 %. Butylphenol did not follow the same trend, the greatest recovery was 6 % and this was again seen in the methanol wash.

C_8

Condition - 5 ml methanol, 5 ml de-ionised water

Sample - 5 ml spiked water (a smaller sample was used to reduce extraction time.)

Wash - 2.5 ml methanol

Dry under vacuum

Elution - 5 ml acetonitrile, 5 ml DCM

All fractions were collected and analysed as before though no analytes were detected with the limit of detection determined to be 12.6 μg / l (ppb).

Diol

Condition - 5 ml methanol, 5 ml de-ionised water

Sample - 10 ml spiked water

Wash - 4 ml de-ionised water

Elution - 10 ml methanol

A 12 % recovery of nonylphenol was observed in the methanol elution step but butylphenol was not found in any of the fractions.

Graphite carbon black

The GCB cartridges contained 250 mg packing material with a capacity of 3 ml, supplied by Supelco (Dorset, UK).

Condition - 3 ml 70:30 DCM / methanol

Sample - 10 ml spiked water

Wash - 5 ml methanol

Elution - 5 ml 30:70 DCM / methanol (pH 1.5), 2 × 5 ml 70:30 DCM / methanol (basic)

The first elution step of 30:70 acidic DCM / methanol produced the highest recovery of nonylphenol within a range of 38-94 % suggesting a reproducibility problem. There was evidence of further nonylphenol elution in the basic elution steps though less than 10 %. There was a slight recovery of butylphenol in the acidic elution but it was less than 10 % and was not evident in the remaining basic elution steps.

C_2

Condition - 3 ml ethyl acetate, 3 ml 50 : 50 methanol / de-ionised water, 3 ml de-ionised water

Sample - 10 ml spiked water

Wash - 3 ml de-ionised water

Dry under vacuum

Elution - 3 ml ethyl acetate, 3 ml 50 : 50 methanol / DCM

Both nonyl- and butylphenol were eluted with the ethyl acetate phase, recovery of nonylphenol was 34 % and butylphenol was 25 % with a small percentage in the methanol / DCM step.

From the preliminary screening of a variety of cartridges there was only one type of packing that was worth investigating further, that being C_2. The other packing materials exhibited varying degrees of recovery for nonylphenol but only one (GCB) gave butylphenol recovery and that was still very low (< 10 %). The C_2 SPE cartridge was optimised by altering solvent volumes and ratios, the optimised method is shown below with calibration of the extraction method found in Chapter 3.

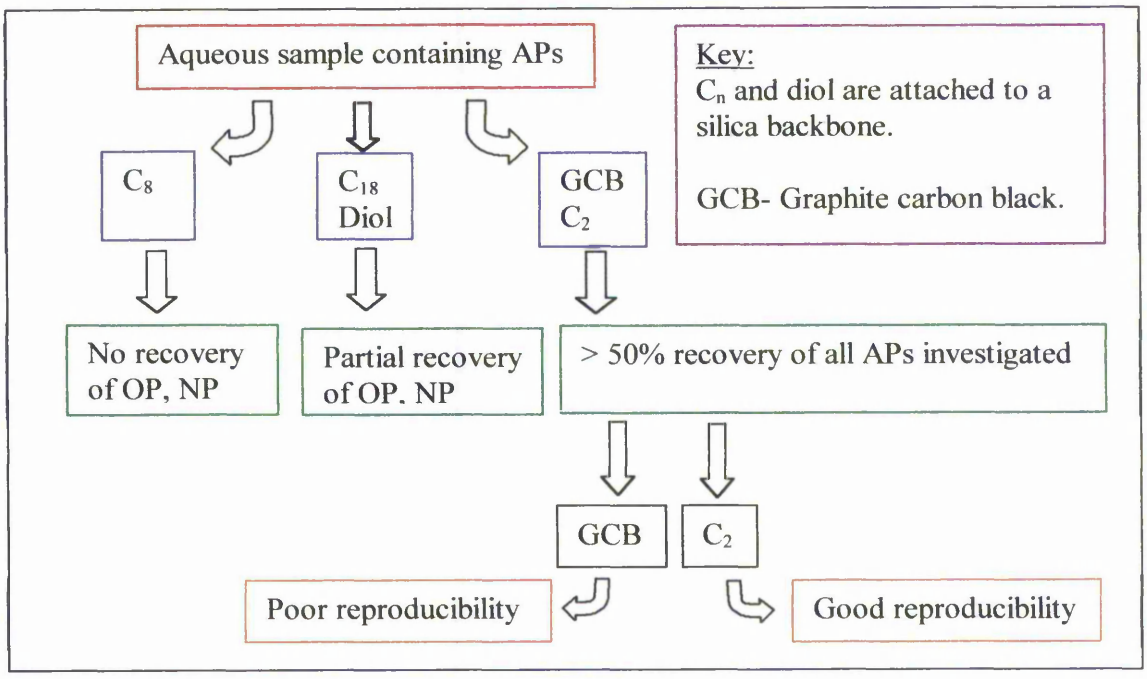


Figure 2.14 *Schematic diagram for choosing a suitable SPE packing material for the extraction of alkylphenols from an aqueous matrix.*

Larger cartridges were employed 500 mg, 6 ml to allow for greater sample throughput, the suppliers were also changed to IST (Hengoed, UK) with greater reproducibility observed.

Condition - 3 ml ethyl acetate, 3 ml 50 : 50 methanol / de-ionised water, 3 ml saline de-ionised water (35 g / l)

Sample - Saline water (35 g / l), volume variable

Wash - 1.5 ml de-ionised water, 1.5 ml 50 : 50 methanol / de-ionised water

Dry under vacuum

Elution - 4.5 ml ethyl acetate

The elution step was collected in a 5 ml graduated flask and was made up to the mark. A portion of this elution liquor is sealed in a glass vial ready for HPLC analysis and in the case of an environmental extract the remainder was stored in a screw top vial in the fridge (< 4°C). The auto sampler vials were supplied by Vials Direct (Macclesfield, UK), other suppliers were tried but a contamination problem with the caps was experienced.

2.4 Alkylphenol extraction from a solid matrix

There were two stages to the extraction of alkylphenols from sediment. Firstly there was the extraction step, followed by the concentration and clean up step. A number of extraction techniques have been employed including soxhlet [Marcomini *et al.*, 1990, Bennie *et al.*, 1997], steam distillation [Giger *et al.*, 1981,], supercritical fluid extraction [Lee and Peart, 1999] and enhanced solvent extraction [Hale *et al.*, 2000]. The technique most suitable for this study was soxhlet extraction, the optimisation of solvent and extraction time are covered in detail in Section 2.4.1.

The next stage prior to analysis was the clean up and concentration step. Methods cited in the literature include column chromatography with florisil [Khim *et al.*, 1999, Sundaram and Szeto, 1981], silica [Bennet and Metcalfe, 1998, Bennie *et al.*, 1997] and size exclusion [Hale *et al.*, 2000], as well as SPE with packings including strong anion exchange and amino phases [Marcomini *et al.*, 2000].

2.4.1 Soxhlet Extraction

Soxhlet extraction is an efficient method of extraction with the repeated recycling of the solvent through the solid matrix (Fig. 2.15).

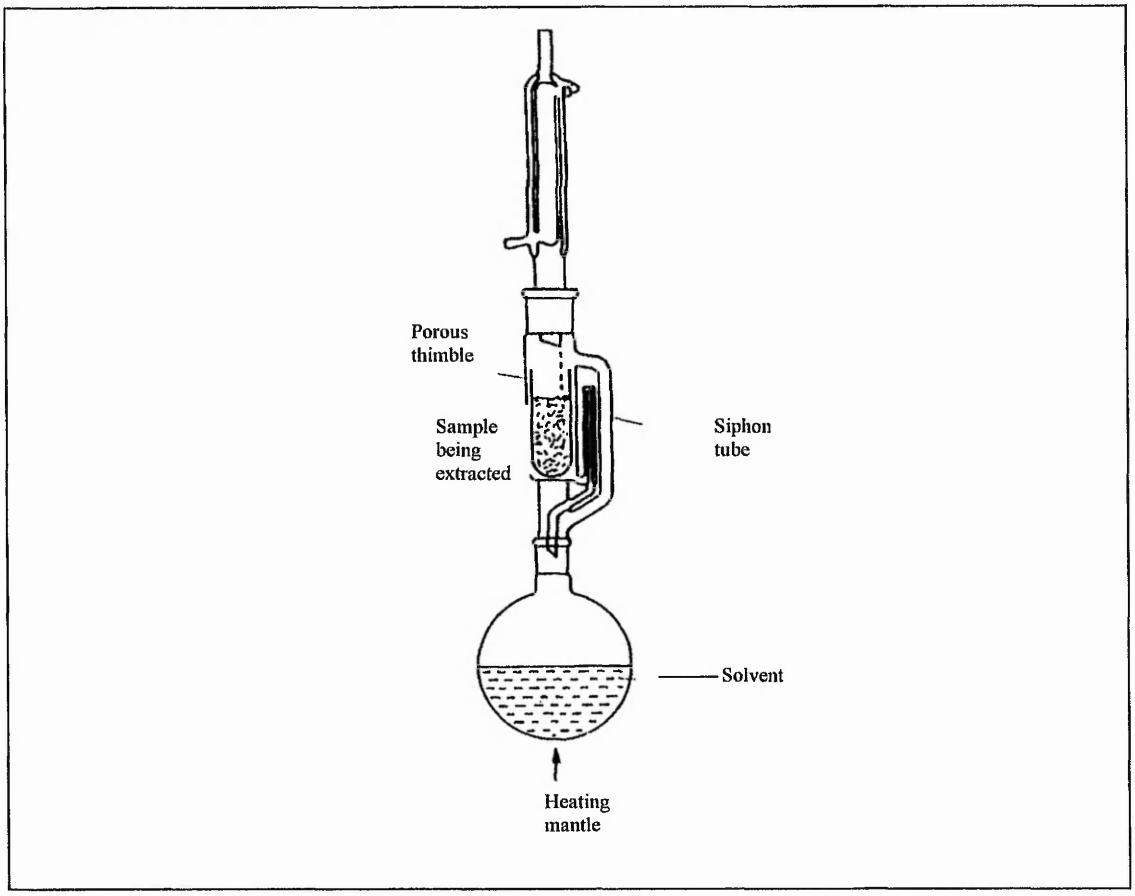


Figure 2.15 *Soxhlet apparatus [Sharp et al., 1989]*

The main factors that need to be optimised for extraction efficiency are solvent type and extraction time, these are discussed in the following sections.

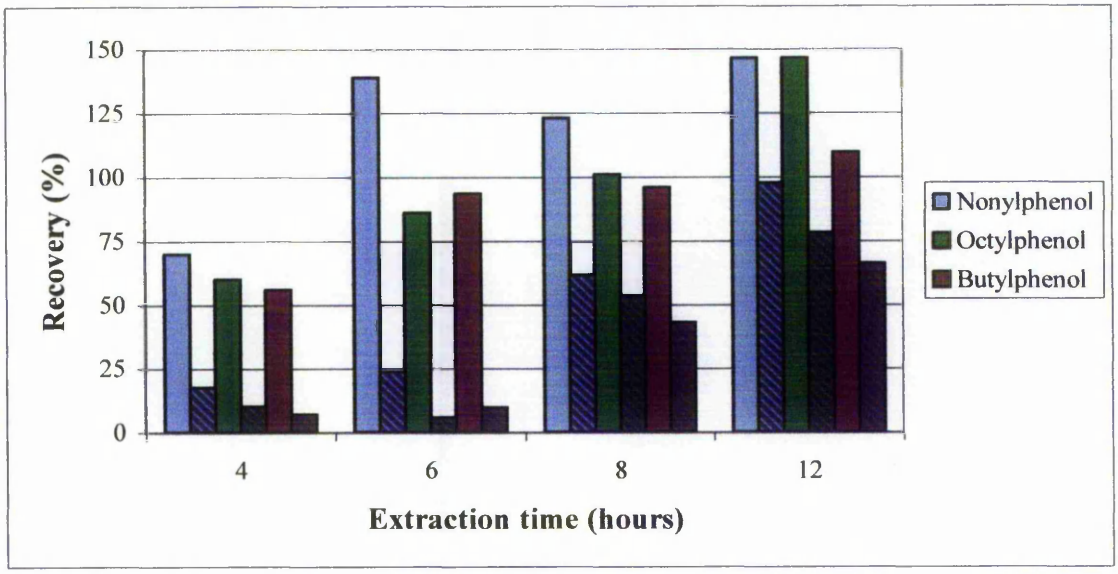
2.4.1.1 Solvent Choice

The method development work was carried out on river sediment as there was no blank alternative, the sample was used for all recovery work. The sediment was spiked at a concentration of 1 mg / l for nonyl- and butylphenol, with the extraction time set at 19 hours, with results summarised in Table 2.4. The spiking of the sediment did not give a true model of sorption and desorption as the spiked analyte will only penetrate the surface of the sediment particles. To achieve greater homogeneity the sediment would need to be left for a number of hours and be subjected to mixing. The method applied in this study was not ideal but did give an indication of extraction efficiency.

Table 2.4 *Recovery data for soxhlet extraction using a range of solvents.*

		Recovery (%)	
Solvent	Reference	Nonylphenol	Butylphenol
Iso-hexane	Marcomini *et al.*, 1990	42.3	10.2
DCM	Chee *et al.*, 1996, Bennie *et al.*, 1997	89	42
Methanol	Voogt *et al.*, 1997	Co-eluting peak	58
Basic methanol pH=10	"	"	6

The results from Table 2.4 indicate some form of contamination with the methanol extraction solvent, therefore only iso-hexane and DCM were investigated further. Duplicate extractions were performed with extraction times of 4, 6, 8 and 12 hours for both solvents.



Firgure 2.16 *Recovery of sediment spiked at 1mg / kg (ppm) with each analyte over various extraction times. The hatched bars are for DCM, plain bars, iso-hexane.*

The results (Fig. 2.16) would suggest that for the greatest recovery in the minimum amount of time then iso-hexane would appear to be the better solvent in comparison to DCM. The optimum extraction time being 6 hours as no significant increase was gained above this extraction time, a number of the extraction recoveries were above 100 % and this is possibly a result of contamination though the source was unknown.

2.4.1.2 SPE concentration and clean up of soxhlet extract.

As with the SPE method development for water analysis a range of packing sorbents were considered. The non-polar nature of the matrix indicated the more polar packing materials such as silica or cyanopropyl. The various sorbents were compared using a standard method shown below with a DCM 1 mg / l spiked standard. DCM was the chosen standard solvent as this experiment was run in parallel to the soxhlet solvent optimisation work.

500 mg, 6 ml (IST) cartridges

Condition - 7.5 ml ethyl acetate, 7.5 ml DCM

Sample - 7.5 ml 1 ppm spiked DCM standard

Wash - 2.5 ml DCM

Elution - 2.5 ml ethyl acetate

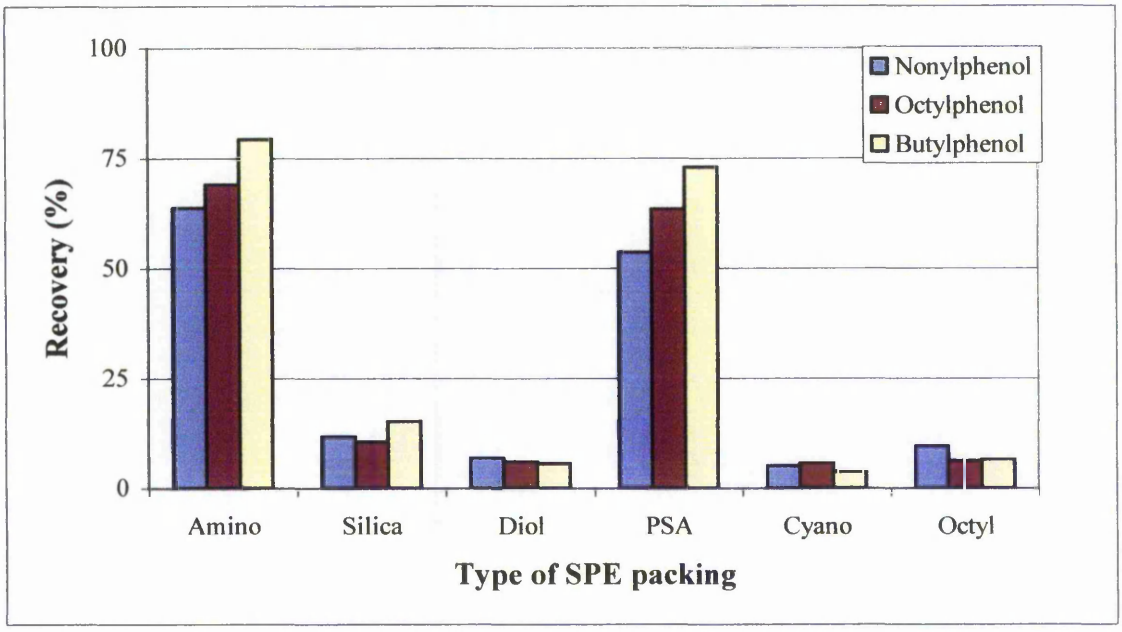


Figure 2.17 *Recovery data for a 1 mg / l (ppm) alkylphenol standard in DCM comparing different SPE packing materials. PSA – ethylenediamine-N-propyl.*

The graph in Fig. 2.17 indicated the highest recoveries were gained using an amino packing sorbent, therefore further studies were carried out on this type of cartridge. The optimised method for the clean up and concentration step was:

Condition - 5 ml MTBE, 3 ml iso-hexane

Sample - 25 ml sample

Wash - 1.5 ml iso-hexane

Elution - 4.5 ml MTBE

The eluent was again collected in a 5 ml volumetric flask and made up to the mark prior to HPLC analysis. The method was calibrated and the results may be found in Chapter 4.

2.5 Summary

The method development work suggests the use of normal phase HPLC as a suitable analytical method for the analysis of alkylphenols, with the use of an amino propyl column (250 × 4.6 mm) and a mobile phase of 70 : 30 cyclohexane / MTBE at a flow rate of 1.5 ml / minute. The use of fluorescence as a detection system was discussed and concluded as the most appropriate method of detection, using 230 nm / 306 nm for excitation and emission wavelengths respectively.

Extraction of alkylphenols from water was explored with a number of techniques, though solid phase extraction using a C_2 packing sorbent presented the best results in terms of extraction efficiency, reproducibility and reliability. The extraction of APs from a solid matrix was more difficult as both an extraction and clean up step had to be developed. Soxhlet extraction using iso - hexane as the extracting solvent was assessed to be the most suitable method of extraction with an aminopropyl SPE cartridge employed for clean up.

With the extraction methods determined for both aqueous and solid matrices, calibration of these procedures needs to be employed and application to environmental samples. These factors are considered in Chapters 3 and 4 with calibration data shown as well as results from the two surveys employed on the Forth Estuary.

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Chapter 3: The Determination of Nonylphenol and Octylphenol in an Aqueous Matrix

3.1 Introduction

A number of extraction and analytical techniques have been employed for the analysis of alkylphenols in environmental waters as shown in Table 3.1. Solid phase extraction would appear to be the method most widely employed for extraction purposes, there seems to be little to choose between the analytical techniques. The limit of detection for the analysis of water samples was around 0.01 μg / l, regardless of which technique was employed.

Table 3.1 *Reported methods for the analysis of alkylphenols in an aqueous matrix*

Sample source	**Extraction**	**Analysis**	**Environmental concentration (μg / l)**	**Limit of Detection (μg / l)**	**Reference**
30 US rivers	Steam distillation	HPLC	<0.11-0.64	0.107	Naylor *et al.*, 1992
6 UK rivers 10 UK Estuaries	SPE, C_{18}	GC-MS	<0.2-30 <0.2-5.8	0.1	Blackburn *et al.*, 1999
River 1994 1995 1996	SPE, GCB	GC-MS	 <0.1-158 <0.1-32 <0.1-8.8	0.01	Davi and Gnudi, 1999
Krka River Estuary, Croatia	Steam dis. /solvent extraction.	NP-HPLC-fluorescence	<0.1-420	0.02	Kvestak *et al.*, 1994
Lao-Jie River, Taiwan	SPE, GCB	GC-MS	1.8-10	Not reported	Ding *et al.*, 1999
Glatt River, Switzerland	Steam dis./ solvent extraction.	NP-HPLC-fluorescence.	0.75-9.5	0.01	Ahel *et al.*, 1996
5 UK rivers 7 UK estuaries	SPE, C_{18}	GC-MS	<0.2-53 <0.08-3.1	0.03	Blackburn and Waldock, 1995
Rhine and Glatt river, Switzerland	Liquid – liquid extraction (hexane)	NP-HPLC	<0.03 – 0.48	0.03	Ahel *et al.*, 2000

3.2 Method calibration and validation using standards

The methods to be employed for the extraction and analysis of alkylphenols in water have been discussed in Chapter 2 and are summarised below.

Water extraction

Solid phase extraction on a C_2 cartridge, elution of the analytes of interest by ethyl acetate. Eluant then subject to analysis.

Analytical method – normal phase HPLC

NH_2, 250 × 4.6 mm column, 5 μm particles. Pre-mixed mobile phase of 70 : 30 cyclohexane / MTBE (methyl *tert.*-butylether) with a flow rate of 1.5 ml / minute, injection volume 50 μl and fluorescence detection (230 nm $_{ex.}$, 306 nm $_{em.}$).

3.2.1 Calibration of the analytical method

Analysis of a nonylphenol, octylphenol and butylphenol standard solution in ethyl acetate were carried out employing the analytical method described in Chapter 2. Each standard was analysed in triplicate and a standard 0.5 mg / l chromatogram is shown in Fig. 3.1. From this chromatogram a number of parameters can be determined, with the most important being that of resolution.

Resolution was calculated below using Eq. 2.8 from Chapter 2.

$$\text{Nonylphenol / Octylphenol} = \frac{11.847 - 10.511}{0.5\,(1.5 + 1.4)} = 0.92$$

$$\text{Octylphenol / Butylphenol} = \frac{13.287 - 11.847}{0.5\,(1.4 + 1.3)} = 1.07$$

These resolution values are a little below the ideal figures though still within an acceptable range.

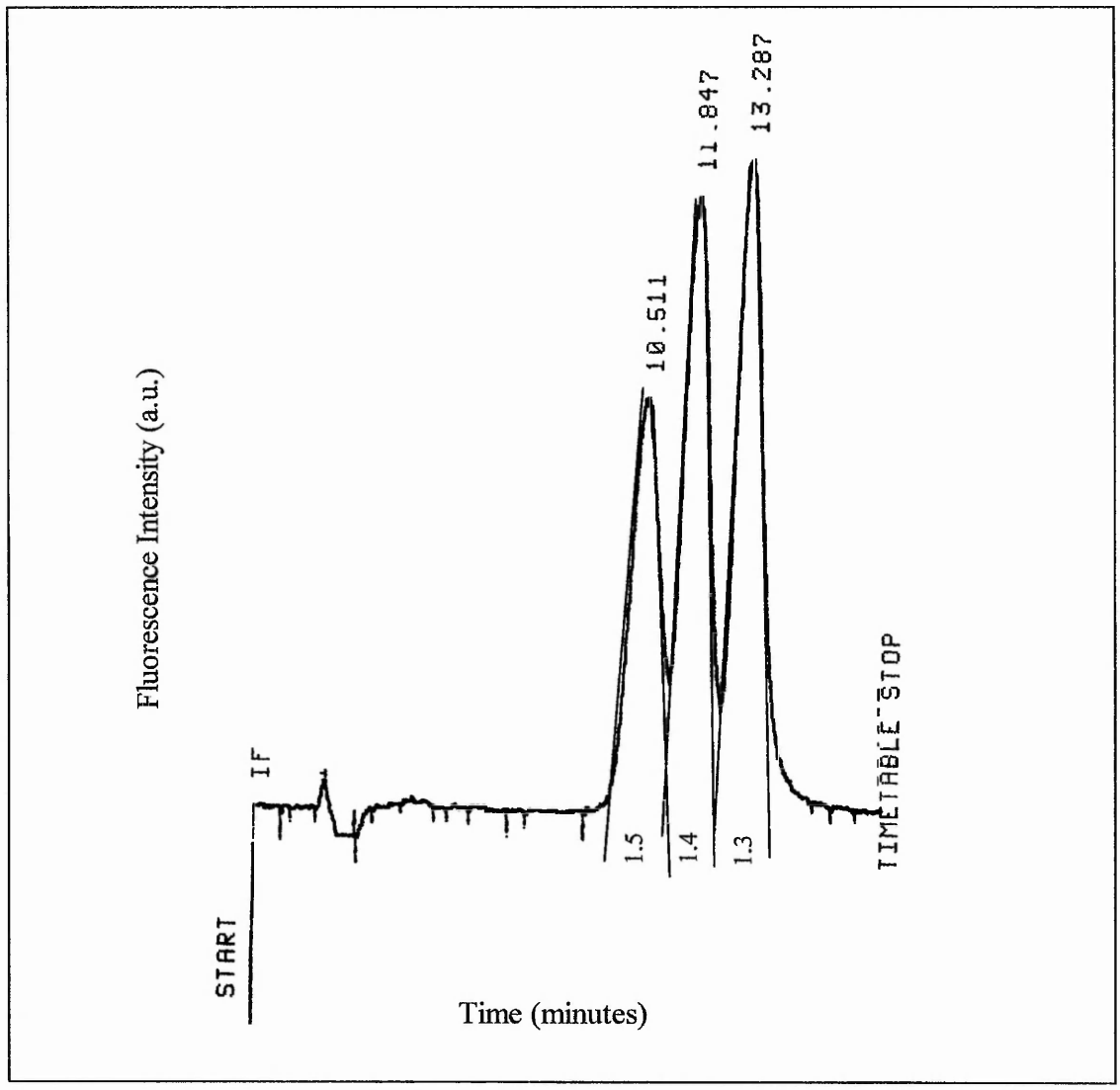

Figure 3.1 *0.5 ppm nonyl-, octyl- and butylphenol standard in ethyl acetate.*

For a range of mixed standards a linear response was obtained over a range of 0.03 mg / l – 1.0 mg / l (ppm) (Fig. 3.2). The limit of detection for the HPLC method was 0.0126 mg / l, defined by a solvent blank plus twice the standard deviation of that blank.

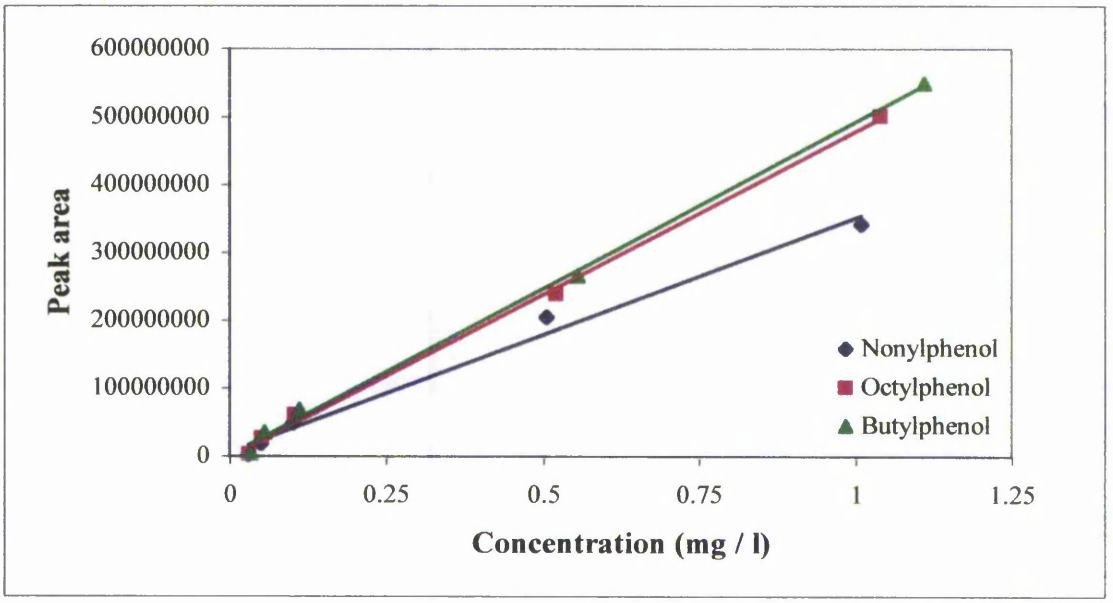

Figure 3.2 *Calibration graph of nonyl-, octyl- and butylphenol standards in ethyl acetate.*

The repeatability of the analytical method was calculated and displayed in Table 3.2, with the RSD (relative standard deviation) values within an acceptable range. There was an increase in RSD as concentration decreased which was expected.

Table 3.2 *Repeatability data for the calibration graph (n = 3)*

Nonylphenol		Octylphenol		Butylphenol	
Conc. (mg/l)	RSD (%)	Conc. (mg/l)	RSD (%)	Conc. (mg/l)	RSD (%)
1.01	0.84	1.04	0.36	1.11	1.30
0.505	2.40	0.52	2.15	0.555	2.73
0.101	6.75	0.104	3.20	0.111	2.60
0.051	2.50	0.052	3.71	0.056	0.05
0.030	4.48	0.031	3.69	0.033	7.30

3.2.2 Calibration of the extraction method

Analyte recovery data was generated using de-ionised water, made to a salinity of 35 g / l with sodium chloride, spiked with nonylphenol, octylphenol and butylphenol at varying concentrations. For the 1 - 0.05 mg / l standards the volume of the sample extracted was 100 ml, this was taken to a final analysis volume of 5 ml, resulting in a concentration factor of 20. The volume extracted for the 0.1, 0.01 - 0.0005 mg / l standards was 500 ml, with a concentration factor of 100. The recovery data is shown in Fig. 3.3.

From the work described in Chapter 2, C_2 was identified as the sorbent that produced the best extraction efficiency in comparison to a variety of sorbents tested, including C_{18}, C_8 and GCB. The C_2 SPE cartridges (6 ml, 500 mg) were conditioned with a 3 ml aliquot of ethyl acetate, 50 : 50 methanol / water and de-ionised water with a sodium chloride content of 35 g / l. All samples were taken to a salinity of 35 g / l to ensure consistency in extraction. The sample was passed through the cartridge and then washed with de-ionised water and 50 : 50 water / methanol before air-drying for an hour. The drying step was essential as the water in the rinsing solvent and the ethyl acetate of the eluting solvent were immiscible. Analyte elution from the cartridge was by the addition of 5 ml ethyl acetate. The cartridge packing allowed large sample volume throughput, permitting good analyte pre-concentration when coupled with a small elution volume.

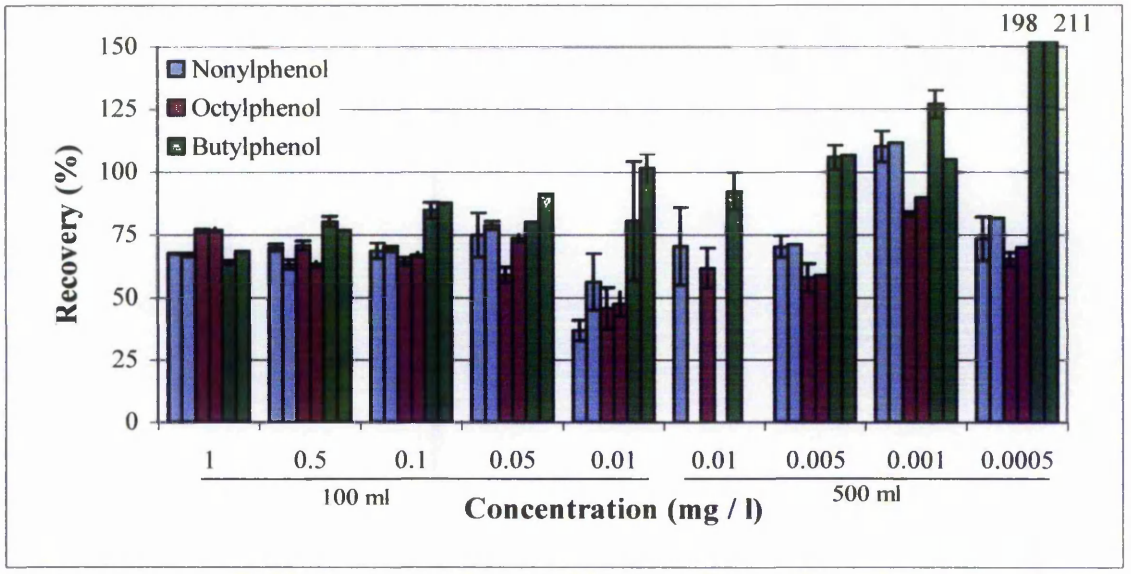

Figure 3.3 *Validation of the C_2 solid phase extraction cartridges using nonylphenol, octylphenol and butylphenol standards in de-ionised water. The volume of each standard extracted is indicated below the concentration.*

Recoveries of between 60 - 100 % were obtained for nonyl-, and octylphenol, RSD values ranged from 0.5% for the 1 mg / l extraction to 13.2 % for the 0.001 mg / l standard. The butylphenol recoveries were between 70 - 90 % to a concentration of 0.005 ppm. The lowest standard extracted (0.0005 mg / l) gave a recovery for butylphenol in excess of 200 %, however the RSD did not increase significantly, suggesting an interference problem. Working at the limits of the analytical method there is greater potential for contamination and this may explain the increase in recovery. This result may ultimately obstruct the use of an internal standard calibration method, though this contamination problem was not systematically observed in the analysis of the environmental samples. The validation of the C_2 cartridges would suggest that recoveries of 75 - 100 % should be expected when applied to environmental samples as expected concentrations are in the range of 0.1 – 1µg / l.

3.2.3 Interlab Validation

The analytical method was validated in the lab using the methods described in the previous section, as well as undergoing external validation by an interlab study conducted by the WRc (Water Research Centre). The published results were generated by solvent extraction with analysis by GC – MS and are compared in Table 3.3 to the results produced by the method described in this chapter. The results signify the validity of the HPLC method and the problems that are observed with GC – MS in terms of quantification.

Table 3.3 *Validation of the analytical method from participation in an interlab study.*

	Target value (ppb)	**TIC**	**MS**	**SIM**	**HPLC-Fluorescence (present study)**
4-octylphenol	**5.12**	5.18 (101.2)	3.7 (72.3)	4.84 (94.5)	8.73 [1] (81.0)
4-nonylphenol	**5.66**	5.68 (100.4)	4.43 (78.3)	5.68 (100.4)	
Tech.-nonylphenol	**9.04**	14.7 (162.6)	2.82 (31.2)	2.98 (33.0)	8.61 (95.2)

TIC – total ion count
MS – mass spectrometry
SIM – single ion monitoring
[1] – combined 4 – nonyl- and octylphenol due to co-elution.
( ) - % recovery

The HPLC values were calculated assuming a 70% recovery, with the reported results comparing well to the target values. These results confirm the accuracy of the method and add confidence to both the extraction and analytical methods. There is a greater amount of variety between the target value of the combined nonylphenol and octylphenol as there was poor resolution between the two peaks and therefore a greater degree of error. The technical grade nonylphenol is the main one used in the manufacture process and is therefore the compound upon which all experiments were carried out.

Analysis of technical grade nonylphenol by GC caused quantification difficulties as a result of the isomeric peaks produced, therefore introducing a high margin of error. For the analysis of 4- octylphenol and 4- nonylphenol the GC methods were comparable to HPLC.

3.3 An initial study on sewage effluent

3.3.1 Sample pre-treatment

The sewage effluent samples were taken from the Stoke Bardolph sewage treatment works in Nottinghamshire. The sewage samples were collected in pre-rinsed 2.5 l Winchester bottles and treated at the point of collection in the way shown below:

Sample 1 - No preservative, no analyte spike.

Sample 2 - No preservative, 0.1 mg / l nonyl-, octyl-, and butylphenol spike (0.05 mg / 500 ml).

Sample 3 - Preservative, no analyte spike.

Sample 4 - Preservative, 0.1ppm nonyl-, octyl-, and butylphenol analyte spike (0.05 mg / 500 ml).

The samples were filtered prior to extraction and taken to 35 g / l salinity with NaCl, these 500 ml saline effluent samples were extracted in triplicate.

3.3.2 Effluent results

The extraction and analysis results from the four sewage effluent samples are found in Table 3.4.

Table 3.4 *Alkylphenol recovery and concentration from sewage effluent and the effect of mercury (II) chloride as a preserving agent.*

Sample	Nonylphenol	Octylphenol	Butylphenol
1	1.53 (0.2) µg / l	bld	bld
2	55.56 (0.5) %	31.62 (1.3) %	71.05 (0.7) %
3	2.04 (0.2) µg / l	bld	bld
4	45.54 (2.6) %	30.92 (1.6) %	69.13 (1.9) %

bld below limit of detection,
( ) RSD, n=3

Table 3.4 clearly indicates the presence of nonylphenol in the effluent sample whether preserved or not, though greater concentrations were observed in the preserved sample. The spiked samples would suggest that the addition of preservative (mercury (II)) made little or no difference to analyte concentration (further discussion in Chapter 5). The repeatability and reproducibility of the method are evaluated in Table 3.5 using the effluent sample 4 (preserved and spiked) data.

Table 3.5 *The % recoveries for sample 4 (preserved sewage effluent extract spiked with nonyl-, octyl-, and butylphenol at a concentration of 0.05 µg / 500 ml).* ( ) % RSD, n = 3.

	1	2	3	Mean (RSD)
Nonylphenol	47.5 (4.4)	42.6 (3.0)	46.6 (1.2)	45.6 (2.6%)
Octylphenol	32.2 (3.8)	29.1 (2.8)	31.4 (1.0)	30.9 (1.6%)
Butylphenol	71.1 (3.3)	67.5 (2.4)	68.8 (1.3)	69.1 (1.9%)

The relative standard deviation for all analytes was below 4.5 % (n = 3) concluding repeatability and reproducibility of the method. The recoveries for nonyl- and octylphenol in extractions from effluent sample 4 were considerably lower than expected in comparison to the validation results (suggested recoveries of approximately 70 %, Fig. 3.3). Butylphenol recoveries would appear to be unaffected (Table 3.5).

There are a number of possible reasons for the decrease in recovery for nonyl- and octylphenol. The two analytes may be susceptible to breakthrough on the SPE cartridge *i.e.* overloading the cartridge may result in premature analyte elution. This is unlikely, as the validation results suggest no analyte loss at a concentration of 1 mg / l, with the % recovery at approximately 70 %. The low recovery would appear to be unrelated to the extraction process, as a greater variation between each set of extractions, and hence high RSD would be expected if this were the case. Another possibility is that the longer chain APs (C_8 or C_9) may have a greater adsorption affinity for particulate matter, and are likely to be removed with the suspended solids *via* the filtration process.

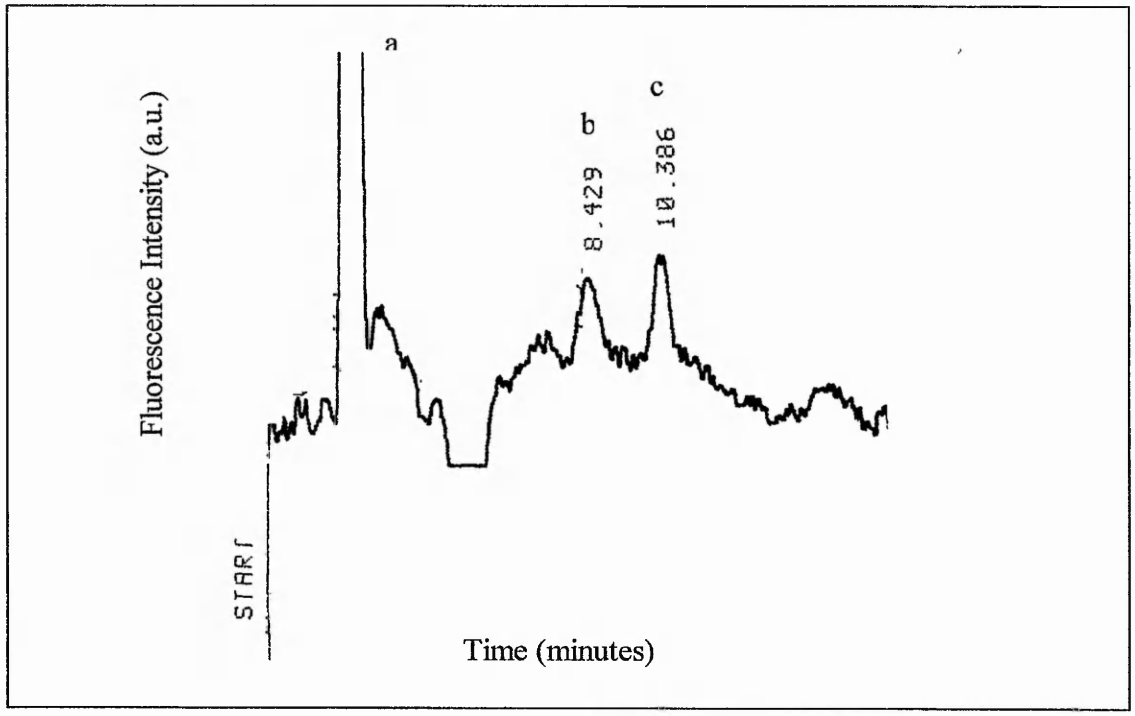

Figure 3.4 *Chromatogram of preserved sewage effluent extract, the significant peaks are assigned, a) solvent peak, b) nonylphenol and c) interference peak.*

Sample 3 was extracted without the addition of the internal standard (butylphenol). The resulting chromatogram (Fig. 3.4) shows the presence of the solvent peak and two other peaks. A standard solution (1 mg / l nonyl-, octyl- and butylphenol) confirmed the peak at 8.429 minutes as that of nonylphenol and quantification was possible by applying an external calibration method. The concentration of nonylphenol in the effluent sample was 2.04 μg / l (ppb) with an RSD of 9.9 % assuming a 100 % recovery. If the extraction efficiency was assumed at 40 % as calculated from the spiked effluent sample (Table 3.4), the recalculated concentration in the effluent was 5.10 μg / l.

3.4 River and Estuarine water analysis

3.4.1 Sample pre-treatment

The environmental water samples were collected in 2.5 l amber acetone washed Winchester bottles using the device shown in Fig. 3.5. The samples were preserved with mercury (II) chloride (approximately 25 mg / l) and stored at room temperature until analysis.

Figure 3.5 *Hand held water sampling device*

Prior to extraction a 2 l portion of the water sample were filtered through a Whatman GF / C filter using Buchner apparatus, the filtered material was analysed and data reported in Chapter 4. 500 ml aliquots were taken to a salinity of 35 g / l, having previously determined initial sample salinity by refractometry. The river and estuarine samples had an internal standard (butylphenol) added for a final concentration of 1 μg / l (0.5 μg in 500 ml). Upon filtration the samples were subject to extraction with two sets of duplicate extractions being performed, this allowed both repeatability and reproducibility to be assessed.

In order to evaluate the adsorption of the internal standard to the particulate matter and to gauge the suspended particles as a possible AP sink, the internal standard was added to the unfiltered sample on the August survey (Section 3.4.3). The sample was then left to equilibrate for 2 hours before filtration and extraction.

Samples were collected in May 2000 during the high spring tide and in August of the same year during the low neap tide from the sampling sites on the Forth Estuary (Fig. 3.6).

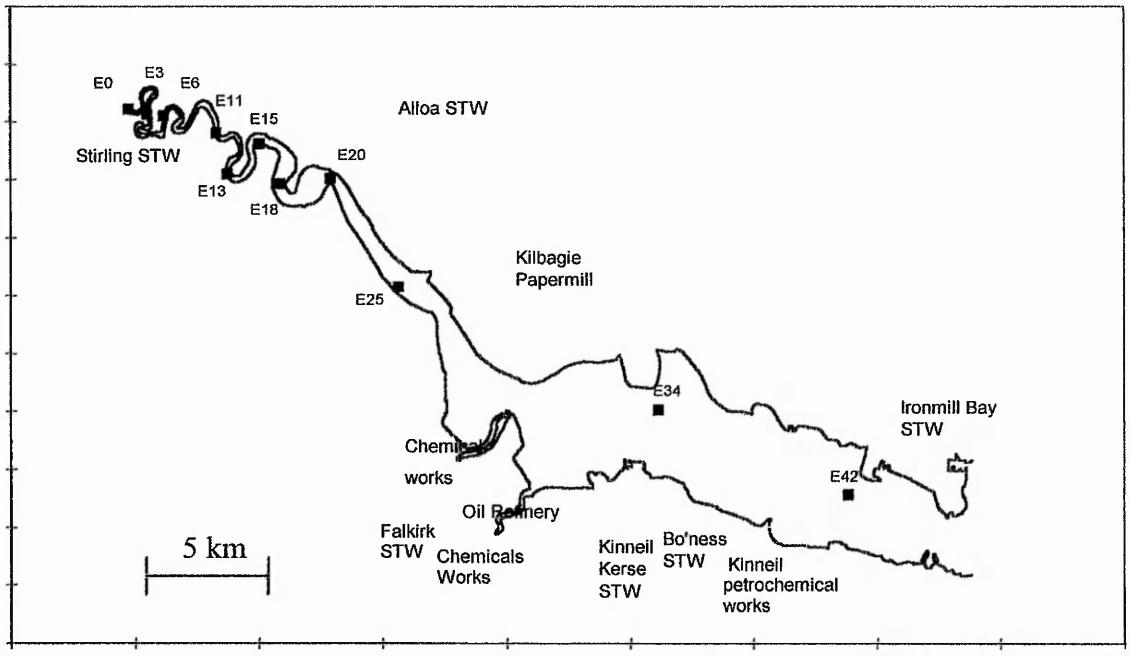

Figure 3.6 *Forth Estuary, STW- sewage treatment works, ■ - sampling sites, the number after E indicates the distance from Stirling (km). Sites of industry are also marked.*

3.4.2 May 2000 survey

Ten sites were sampled for the May survey and the water quality parameters are shown in Table 3.5. This survey found that levels of nonyl- and octylphenol were below the limit of detection *i.e.*, less than 0.13 μg / l. However, the recovery data on butylphenol was encouraging as the results in Table 3.5 indicate.

Table 3.5 *Environmental water quality data and internal standard recovery results for May 2000 from the Forth Estuary.*

Sample site	Salinity (g / l)*	pH*	Turbidity (NTU)*	Butylphenol Recovery (%)				Mean Recovery (%)	RSD
E42+	30	8.11	11	15	12	-	-	13.5	2.1
E34+	27	8.15	45	48	46	82	-	58.7	20.2
E25+	25	8.03	77	-	83	78	-	80.5	3.5
E20	13	7.71	82	107	102	104	100	103.3	2.9
E15	2	7.62	63	96	95	103	-	98.0	4.4
E13	0	7.66	172	79	104	109	101	98.3	13.3
E11	0	7.72	212	115	-	109	-	112.0	4.2
E6	0	7.76	234	91	87	91	91	90.0	2.0
E3	0	7.85	145	113	108	106	119	111.5	5.8
E0	0	-	-	117	121	98	-	112.0	12.3

* data from the SEPA water quality survey
NTU nephelometric turbidity units
\+ unfiltered sample

The first three samples were not filtered prior to extraction (E42, E34 and E25). However, the consequent increase in extraction time as a result of clogging the SPE cartridge prompted the filtration of the remaining samples. The unfiltered samples show evidence of decreased recovery in comparison to the filtered samples. Alkylphenols have an affinity for particulate material [Johnson *et al.*, 1998] and sorption of APs to particles during the extraction stage was the possible cause of the decrease in recovery of the butylphenol internal standard in sample E42, 34, 25. The filtered samples had the internal standard added after sample filtration and resulting recoveries were > 90%, these results were confirmation of BP sorption onto the particulate matter. The filtered samples gave consistent recoveries of butylphenol with low RSD values indicating precision of the extraction method.

3.4.3 August 2000 survey

Low neap tide samples were collected in August 2000 from the same 10 sampling sites as the May survey. There was a large difference in water volume of the estuary between the two survey dates, with water levels at their annual low, therefore effectively equal to a natural concentration step for contaminants / effluents introduced to the estuary. The water quality parameters are displayed in Table 3.6 with the environmental concentrations of nonyl- and octylphenol and the recovery data for butylphenol.

Table 3.6 *Water quality data with nonyl- and octylphenol concentrations and butylphenol recovery results for August*

Sample site	Salinity*	pH*	Turbidity (NTU)*	Nonylphenol (µg / l)	Octylphenol (µg / l)	Butylphenol (% recovery)
E42	32	7.97	2.5	nd	nd	55.2 ± 4.7[c]
E34	30	7.99	18.8	nd	nd	107.4 ± 17.4[c+]
E25	25	7.89	35.0	0.17 (0.08)[b]	0.06[a]	102.4 ± 6.1
E20	16	7.63	65.6	0.70 (0.57)[c]	0.39 (0.37)[b]	169.7 ± 68.9[+]
E18	15	7.56	49.5	1.90 (1.16)	1.28 (0.6)	380.4 ± 158.7[+]
E15	5	7.51	37.9	0.31 (0.25)	0.18 (0.05)[c]	109.3 ± 22.6
E13	5	7.45	130.9	0.79 (0.63)	0.18 (0.05)[c]	162.3 ± 70.4
E11	0	7.48	122.2	0.14 (0.10)	0.33 (0.17)	112.7 ± 10.0[b]
E6	0	7.45	308.5	1.10 (0.46)[b]	0.96 (0.29)[b]	158.3 ± 78.8[c+]
E3	0	7.41	405.6	0.33 (0.13)	0.17 (0.11)	70.2 ± 7.0
E0	0	7.31	113.7	1.13 (0.83)	0.40 (0.03)[c]	128.8 ± 27.7[+]

( ) standard deviation, n=4
a- n=1
b- n=2
c- n=3
+ - interference present

* - data supplied by SEPA
nd - not detected

There was a degree of variation between the four extractions within each sample set (Table 3.6), this was possibly a result of error as a consequence of low level analysis, with an increase in baseline noise. One way to improve the variation would be to increase the volume of water extracted. Some exceptionally high butylphenol recovery values were explained by the presence of an interference peak near the internal standard (Fig. 3.7). The interference peak was broad in comparison to the butylphenol peak and would suggest that the shoulder of this peak was possibly integrated as part of butylphenol by the instrumentation. This interference was not present in all samples and was undetermined, the samples affected by this interference are marked in Table 3.6.

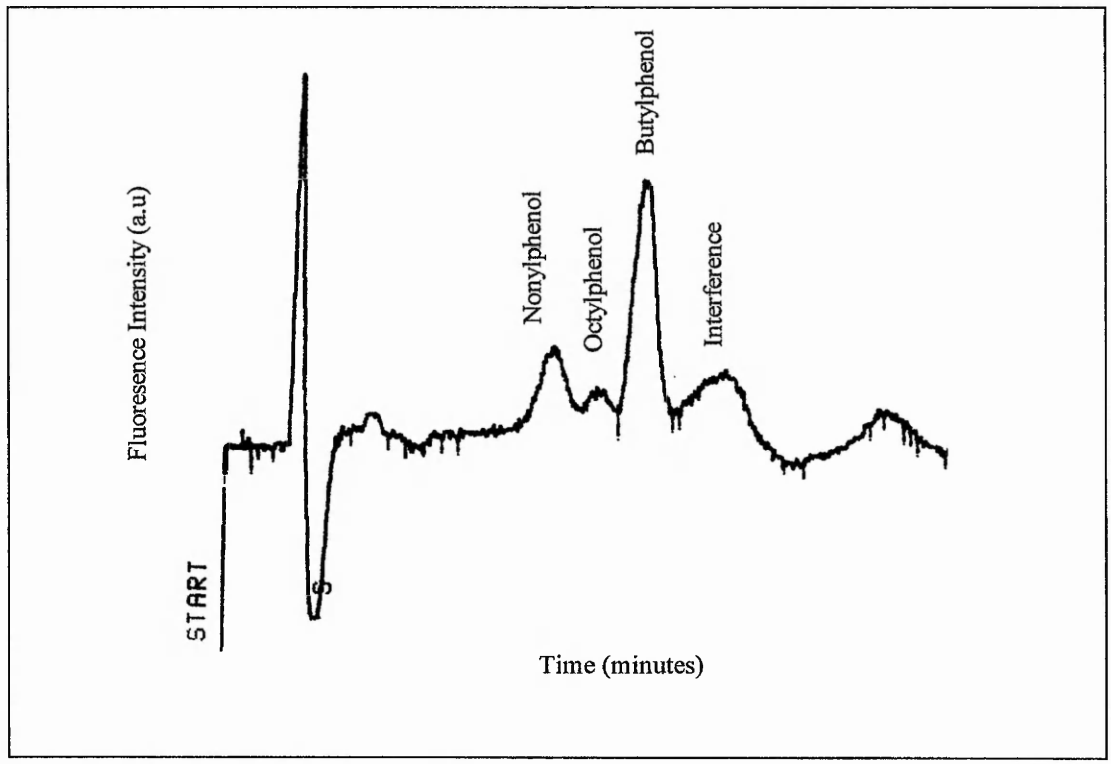

Figure 3.7 *Chromatogram of E0 August survey, the peaks are labelled as the analytes and interference.*

For all but one of the sample sites the concentration of octylphenol was below that of nonylphenol, confirming the greater abundance of nonylphenol in the environment. Sites E18 and E6 exhibited higher concentrations of nonyl- and octylphenol in comparison to the other sites, one explanation may be the presence of sewage treatment works close to the sampling sites. Sites E42 and E34 had concentrations below the limit of detection, this is likely to be a consequence of greater dilution of contaminants as these sampling sites were in the main body of the estuary.

3.4.4 Discussion of results

There is a striking difference between the May and August surveys, with analyte concentrations below the limit of detection for May but present in the August survey. There are a number of possible reasons for these results, the main one being that of water volume. The difference in water volume was calculated in 1978 from Stirling to the Forth Road Bridge with a change from 5.59×10^8 m^{-3} at high water to 3.2×10^8 m^{-3} at low water (SEPA, 1978), this does not equate to seasonal changes or spring – neap differences but it does give an indication of water volume changes. The natural concentration step could therefore be as great as 50 % and would offer a possible reason for analyte presence in the August survey.

Another reason that should be considered is that of temperature, water temperature for May was between 9.2 - 13.5 °C, whereas August temperatures were in the range of 14.8 – 17.1 °C, these small differences may affect the solubility of the analytes of interest. With greater solubility at higher temperatures, the increase in alkylphenol concentrations in the August survey may be explained.

Salinity is also a factor that may affect alkylphenol concentration in the water column (Fig. 3.8)

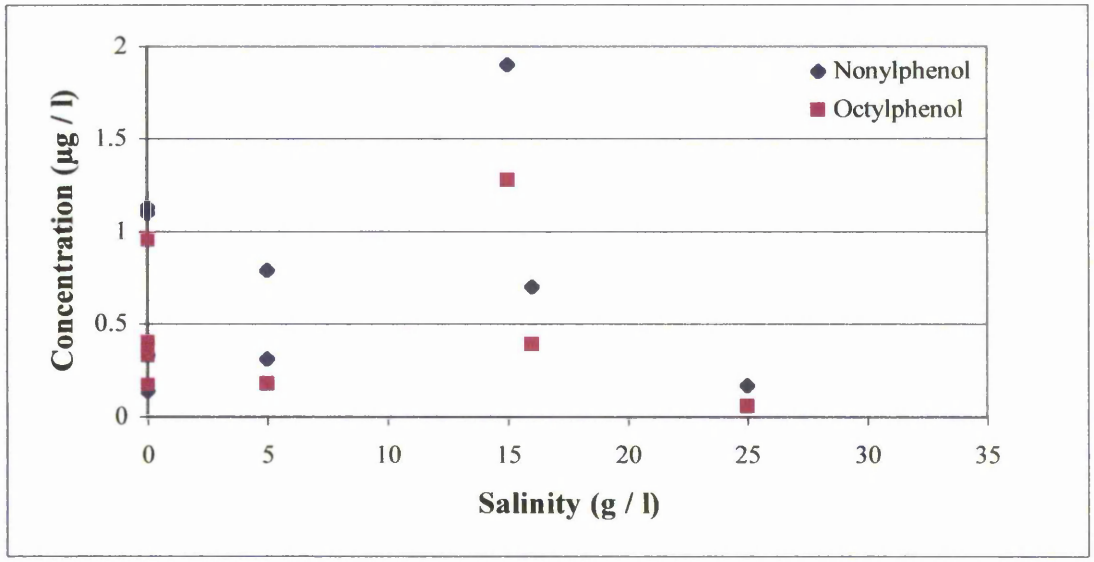

Figure 3.8 *Nonylphenol and octylphenol concentrations against salinity*

Figure 3.8 indicates a possible trend between salinity and alkylphenol concentration. If the two points above 1.3 are excluded then the R^2 values are 0.1138 for nonylphenol and 0.1683 for octylphenol. Results suggest that at higher salinity there is a decrease in concentration of the alkylphenols, this trend may however be the result of an increase in water volume rather than an increase in salinity. Further work in this area is needed before conclusions can be drawn with confidence.

3.5 Summary

Confidence in the extraction and analytical methods were high as a result of rigorous validation, with recoveries of all three alkylphenols around 100 %. The integration of the interference as part of the butylphenol peak made internal calibration impossible, with environmental concentrations of nonyl- and octylphenol calculated by an external calibration method. There was an absence of the target analytes from the May survey, the reason being, greater volume of water resulting in greater dilution of the analytes. The August survey produced alkylphenol concentrations above the limit of detection, with results comparable to other UK river surveys.

In summary, concentrations of nonyl- and octylphenol in the Forth Estuary were measured from < 0.13 - 1.9 ppb (μg / l) (Table 3.6). These levels are consistent with those in the literature for UK Rivers and estuaries, with the River Aire < 0.2 - 30 μg / l and the Tees Estuary < 0.2 - 5.8 μg / l (Blackburn *et al.*, 1999). The concentrations found in the August water survey are lower than concentrations found to induce an oestrogenic response in aquatic organisms, the threshold for vitellogenin induction has been found to be 10 μg / l for nonylphenol and 3 μg / l for octylphenol (Jobling *et al.*, 1996). However, if bioaccumulation is considered (Ahel *et al.*, 1993) with bioconcentration factors of 1,300 for stickleback (Ekelund *et al.*, 1990) and 280 for Atlantic salmon (McLeese *et al.*, 1981) then the measured environmental concentrations are potentially within the limits to induce vitellogenin production in male or juvenile female fish.

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Chapter 4: The Determination of Nonylphenol and Octylphenol in a Solid Matrix

4.1 Introduction

This chapter will introduce the various methods available for the extraction and analysis of sediment and suspended solids and the environmental concentrations that have been determined from the application of these methods. The extraction and analytical methods to be applied in this study will be outlined again, with calibration and validation results reported and limits of detection calculated. These methods will then be applied to environmental samples collected from two surveys of the Forth Estuary, with the results discussed and compared with other estuarine systems.

4.1.1 Analysis of sediments

In the analysis of alkylphenols in sediment a number of extraction and pre-concentration steps have been reported (Table 4.1). Soxhlet extraction appears to be the most popular method of extraction, though it is likely to be replaced by super critical fluid extraction as a result of shorter extraction time and reduced solvent volumes. A majority of the clean up and concentration methods are chromatographically based, whether it is SPE or a packed column. Analysis of the sediment extracts is favoured by GC - MS with the lowest detection limit of 0.3 pg / g (0.3 ng / kg) achieved by using a negative-ion chemical ionisation method. The HPLC results do however indicate that it is a viable method with detection limits being comparable to most of those achieved by GC - MS. The concentrations of alkylphenols in the river and estuarine sediment ranged from < 4-72,000 μg / kg with the highest concentrations reported in the Great Lakes of the United States and Canada.

Table 4.1 *Present methods of sediment analysis.*

Location	Extraction	Clean-up	Analysis	Env. Conc. (µg / kg)	L.O.D (µg / kg)	Reference
30 US rivers	Steam dis.	-	HPLC	<2.9-2960	2.9	Naylor *et al.*, 1992
River: Aire, UK Mersey, UK	Sonication, centrifuge	SPE C_{18}	GC-MS	 <100-1,500 260-520	100	Blackburn *et al.*, 1999
Venice Lagoon, Italy	Soxhlet (hexane)	SPE NH_2	NP-HPLC-fluor.	5-42	1	Marcomini *et al.*, 1990
Estuary: Tyne, UK Tees, UK	Steam dis.	Alumina column	GC-FID/ GC-MS	 30-80 1600-9050		Lye *et al.*, 1999
Nile estuary, Barcelona coast, Spain	Soxhlet 2:1 (DCM/MeOH)	Alumina column, Pentafluoro benzyl deriv.	GC-ECD	19-44 6-69	2.9pg/gECD 1.4pg/gEI-MS 0.3pg/gNCI	Chalaux *et al.*, 1994
Virginia, USA	Soxhlet, enchanced solvent	NP-LC	GC-FID	<5-12400	5	Hale *et al.*, 2000
Humber Estuary, UK Ouse, UK Aire, UK Swale, UK Calder, UK Don, UK Trent, UK	Supercritical fluid extrac.	-	GC-MS	 9-64 14 39-69 31 12 6	0.5	Long *et al.*, 1998
Great Lakes, USA, Canada	Supercritical fluid	Acetylation, silica gel column	GC-MS-SIM	NP <46-37,800 OP <1-23,000	46 1	Bennett and Metcalfe, 1998
Lake Shihwa, Korea	Soxhlet (DCM)	Florosil column	RP-HPLC	NP 20.2-1820 OP 4.69- 50.5	1	Khim *et al.*, 1999 a
Great Lakes USA/Canada	Supercritical fluid	Silica gel	EI-GC-MS	NP 170-72,000 OP <10-1,800	100	Bennie *et al.*, 1997

GC	gas chromatography	HPLC	high performance liquid chromatography
HRGC	high resolution GC	NP	normal phase
FID	flame ionisation detection	RP	reverse phase
ECD	electron capture detection	UV	ultraviolet detection
MS	mass spectrometry	Fluor	fluorescence detection
EI	electron ionisation		
NCI	negative-ion chemical ionisation		
SIM	single ion monitoring		

4.1.2 Suspended solids analysis

There are less data available on the suspended solids as shown in Table 4.2. There seems to be no dominant method for extraction, though GC-MS is favoured for analysis, with limits of detection ranging for 0.5 – 15 µg / kg. The environmental data demonstrate the range of concentrations that can be found in a single water course and the comparability of the data between the different river systems, with the greatest concentration of 3,540 µg / kg being recorded from a sample collected in Tokyo Bay, Japan.

Table 4.2 *Present methods for the analysis of nonylphenol and octylphenol in suspended solids*

Location	**Extraction**	**Clean-up**	**Analysis**	**Env. conc. (µg / kg)**	**L.O.D. (µg / kg)**	**Reference**
Fraser River, Canada	XAD-2		GC-MS	35-87 (0.92-2.39 ng / l)		Sekela *et al.*, 1999
UK rivers Ouse Aire Swale Calder Don Trent	Supercritical fluid extraction	-	GC-MS	 38-521 48-402 32-71 205-339 458-1,048 29-107	0.5	Long *et al.*, 1998
Tokyo Bay, Japan	Soxhlet (DCM)	Silica gel	GC-MS	NP 3,540 OP 330	15 3	Isobe *et al.*, 2001

4.2 Method calibration using standards

The methods to be employed for sediment extraction and analysis in this present study have been discussed in Chapter 2 and are summarised below.

Sediment extraction

Soxhlet extraction over 6 hours into iso-hexane, followed by NH_2 solid phase extraction, with elution of the analytes of interest into MTBE.

Analytical method

Normal phase HPLC using a 250 × 4.6 mm NH_2 column, 5μm particles. Pre-mixed mobile phase 70:30 cyclohexane / MTBE with a flow rate of 1.5 ml / minute, injection volume of 50 μl and fluorescence detection (230 $nm_{ex.}$, 306 $nm_{em.}$).

4.2.1 Calibration of the analytical method

Analysis of the alkylphenol standards was carried out using the instrumentation described in Chapter 2. The standards were placed in crimp top glass vials in the autosampler and 50 μl aliquots were injected onto the column. Analysis was conducted in triplicate. A 0.1 mg / l standard chromatogram of nonyl-, octyl- and butylphenol in methyl-tert.- butyl ether (MTBE) is shown in Fig. 4.1. From this chromatogram theoretical parameters of the column were assessed the most important being that of resolution. Resolution was calculated using Eq. 2.8.

$$\text{Nonylphenol/ octylphenol} = \frac{12.015 - 10.388}{0.5\,(1.4 + 1.0)} = 1.35$$

$$\text{Octylphenol/ butylphenol} = \frac{13.434 - 12.015}{0.5\,(1.0 + 1.2)} = 1.29$$

These values were between the ideal values of 1.2 and 1.5 discussed in Section 2.1.2.2.

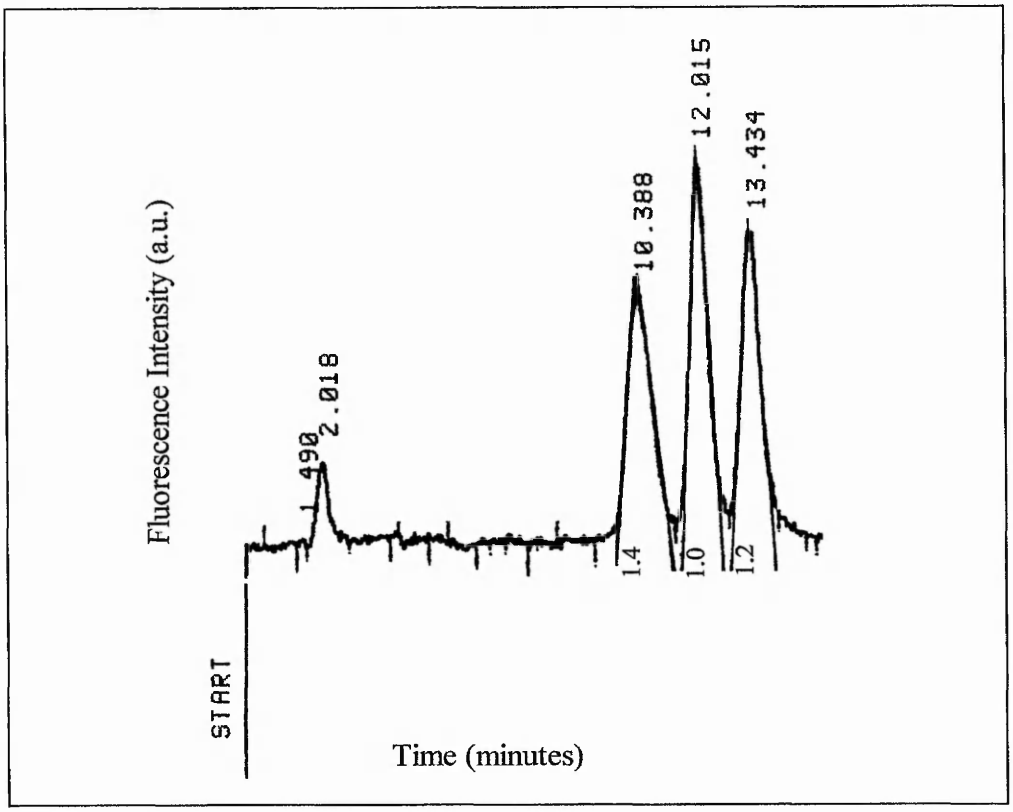

Figure 4.1 *0.1 mg / l standard of nonylphenol, octylphenol and butylphenol in MTBE. Peak identification are in minutes, while peak widths are annotated in cm.*

A calibration graph was generated by the analysis of a range of concentrations of a mixed alkylphenol standard in MTBE (Fig. 4.2). The calibration graph indicates linearity from 0.01- 1.5 mg / l, the limit of detection was calculated to be 0.008 mg / l based on the signal produced from a blank run of MTBE, plus twice the standard deviation of that run.

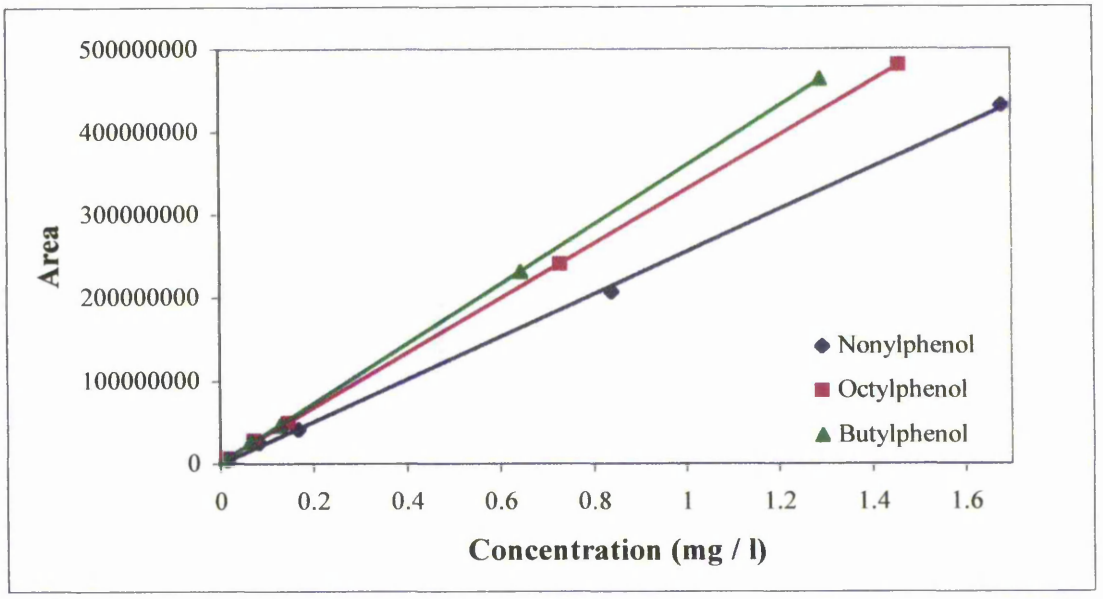

Figure 4.2 *A calibration graph of nonylphenol, octylphenol and butylphenol standards in MTBE.*

Each concentration was analysed in triplicate to assess the repeatability of the analytical method (Table 4.3). The results would indicate that the method was repeatable over the linear range and does not appear to be effected by concentration, which is what might be expected.

Table 4.3 *Repeatability data for the calibration standards, (n=3).*

Nonylphenol		Octylphenol		Butylphenol	
Conc. (mg/l)	RSD (%)	Conc. (mg/l)	RSD (%)	Conc. (mg/l)	RSD (%)
1.68	2.77	1.46	2.48	1.29	2.94
0.84	11.34	0.73	6.4	0.645	9.17
0.168	0.75	0.146	6.0	0.129	3.21
0.084	10.67	0.073	10.1	0.0645	5.54
0.0168	0.21	0.0146	26.2	0.0129	7.85

4.2.2 Validation of the extraction method

The SPE method was validated using a mixed standard of nonyl-, octyl, and butylphenol at a range of concentrations in iso-hexane. The method employed for the validation was developed in Chapter 2 and is summarised below:

Using a 6 ml, 500 mg NH_2 cartridge (IST),

Condition - 5 ml MTBE, 3 ml iso-hexane

Sample - 25 ml iso-hexane standard

Wash - 1.5 ml iso-hexane

Elution - 4.5 ml MTBE

The eluant was collected in a 5 ml volumetric flask and diluted to 5 ml. The validation was employed over a range of 0.001 - 5 mg /l and each concentration was extracted in duplicate sets of 2 in order to determine repeatability and reproducibility (Fig. 4.3).

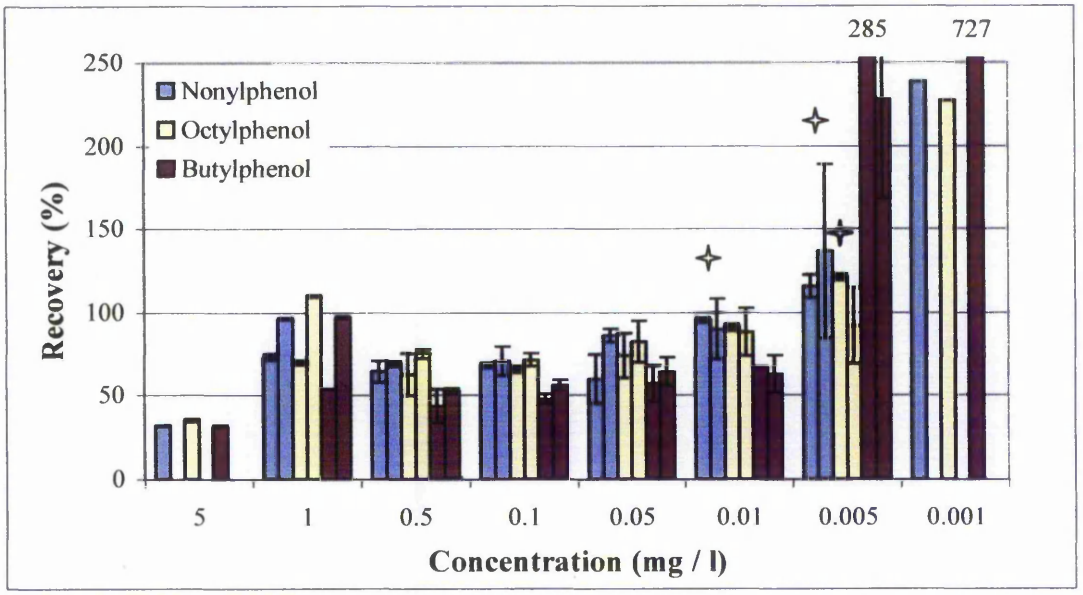

Figure 4.3 *NH_2 calibration graph, there are two data sets for each extraction in order to assess reproducibility within a sample set and repeatability between a sample set. (✧ RSD greater than 15 %).*

There is low recovery (below 40 %) for all 3 analytes at a concentration of 5 mg / l, this is possibly caused by an insufficient volume of solvent to remove all bound analytes. Another potential cause for low recovery would be breakthrough from overloading the sorbent, this possibility was ruled out since a portion from both the sample and the wash eluent was analysed and confirmed no analyte presence. To improve extract recovery at 5 mg / l a greater volume of solvent could be added, though this would result in a smaller concentration step. Figure 4.3 also indicates good recoveries of around 80 % between 1 – 0.01 mg / l for all three analytes, butylphenol recoveries were a little lower and this could be a consequence of greater absorption affinity for the packing sorbent than the other analytes of interest. The retention affinity for the HPLC column (also NH_2) has lead to this conclusion as butylphenol is the last of the three peaks to elute from the column, it is therefore not unreasonable to assume that the same will be true for the SPE cartridges.

At concentrations of 0.005 and 0.001 mg / l the recovery of butylphenol exceeded 700 % and both nonyl- and octylphenol beyond 200 % recovery. These erroneous recoveries are thought to be the result of contamination and may signal the limit of the extraction method. The source of the contamination was unclear though a number of methods were employed in an attempt to eliminate the problem. These included acetone and acid washing all glassware in an attempt to remove all organic matter that may have become adsorbed onto the surface of the glass. The source of the contamination could not be identified or removed and was therefore accepted as background levels. Recent research has identified certain types of latex gloves to contain low levels of some alkylphenols and this could be one of the most likely candidates for the contamination [Voogt *et al.*, 2000].

The repeatability of the SPE method for the duplicate extractions were good, with a majority of the RSD values being below 15 %, those with values greater than 15 % are marked in Fig. 4.3. The reproducibility of the method between the 2 runs was less than 10 % for all but one set of results between 0.5 – 0.01 mg / l. The results for 1 ppm indicate a problem, possibly with the cartridge as all 3 analytes from the 2nd extraction exhibited a greater recovery, though the repeatability (RSD) remained low. The other extraction set that experienced reproducibility problems was that of 0.005 mg / l. There would appear to be no consistent trend for the three analytes as was observed for 1 mg / l and this would suggest random error, and may be a result of working at the limit of the method as mentioned previously.

The soxhlet extraction procedure was optimised in terms of extraction time and solvents, as described in Section 2.4.1.1. A true validation of the soxhlet extraction method was not possible as no blank sediment sample was available. In all extractions a butylphenol spike was added and this was used as an indicator of alkylphenol recovery.

4.3 River and estuarine sediment analysis

4.3.1 Sample pre-treatment

The sediment samples were collected from the Forth Estuary for both the May and August surveys using a hand held grab (Fig. 4.4). Not all sites were suitable for the sampling of sediments, the grab may not have been able to take a sample as a result of the stony composition of the estuary bed or the water may have been too deep for the grab to take a sample. However, where possible a sample was collected.

Figure 4.4 *Hand held grab used for sediment sampling.*

The sediment samples were stored in solvent rinsed glass jars with a sheet of aluminium foil separating the lid from the sediment beneath to reduce the potential for contamination. The sediment samples were stored in a freezer (<-5°C) until analysis. Prior to extraction the sediment samples were allowed to defrost for 24 hours, the samples were then dried in an oven at 60°C. The samples were ground using a pestle and mortar and sieved through a 500 μm sieve. A 15 g sample from the prepared sediment was then weighed into the pre-rinsed thimble and spiked with a known concentration of the internal standard (BP), these samples were then left to equilibrate over night prior to extraction.

Having extracted the sediment for 6 hours in iso-hexane, the liquor was cooled and collected in a 100 ml flask and taken to 100 ml either by the addition of iso-hexane or by drying down under nitrogen. A 25 ml aliquot of the extraction liquor was in turn passed through SPE in duplicate so that repeatability of the SPE method could be determined. The results for the extraction are shown in Section 4.3.2.

The environmental concentrations were calculated using an external calibration method, as discussed later in Section 4.3.2. With each set of results a 0.5 mg / l standard of NP, OP and BP in MTBE were run and a response factor calculated for each analyte (Eq. 2.11). The response factor may then be applied to Eq. 2.12 to obtain the concentration of the alkylphenols present in the sample after extraction, to calculate the original concentration the concentration factor is needed. The concentration factor for this cleanup step is shown below, with the internal standard concentration used as an example.

0.75 ml of a 1.25 μg / ml butylphenol standard added to 15 g of sediment

0.938 μg in 15 g	- amount of BP in the original sample prior to extraction
0.938 μg in 100 ml	- amount of BP in the soxhlet extract
0.235 μg in 25 ml	- amount of BP and volume subject to SPE
0.235 μg in 5 ml	- amount of BP and final volume resulting from SPE eluant
0.047 μg in 1 ml	- the anticipated concentration upon HPLC analysis

The HPLC value is multiplied by the Rf, then by 20 to give the concentration in the original 15 g sample or by 1.33 to give the concentration in 1 g. All calculations assume a 100 % extraction efficiency.

4.3.2 Sediment results

From the May 2000 sampling survey of the Forth Estuary, samples from six sites were extracted. Four sediment samples were collected from the estuary in the August survey. The limit of detection for the extraction and analytical methods was calculated at 10.2 μg / kg (ppb), based on a blank run plus twice the standard deviation of the blank run. The composition of the sediments varied along the estuary as shown in Table 4.4.

The organic carbon content of the sediment samples were determined using the following method.

Approximately 1 g of sediment was accurately weighed into a crucible, the procedure was repeated for all samples. The crucibles were then placed in a furnace oven set at 300 °C for 18 hours. Upon cooling the crucibles were re-weighed and the loss was calculated and presented as a percentage in Table 4.4.

Table 4.4 *Composition of the estuary sediments.*

	Sediment composition			
Estuary site	**May**	**OC %**	**August**	**OC %**
E34	Fine particles	7.1	Fine particles	nd
E25	-		Sand /small stones /fine particles	1.75
E18	Sand / small stones	nd	Sand /small stones	nd
E15	Fine particles	16.4	-	
E11	Fine particles	8.0	-	
E6	Fine particles	2.6	-	
E3	-		Fine particles	1.0
E0	Sand / small stones	nd	-	

OC - Organic carbon

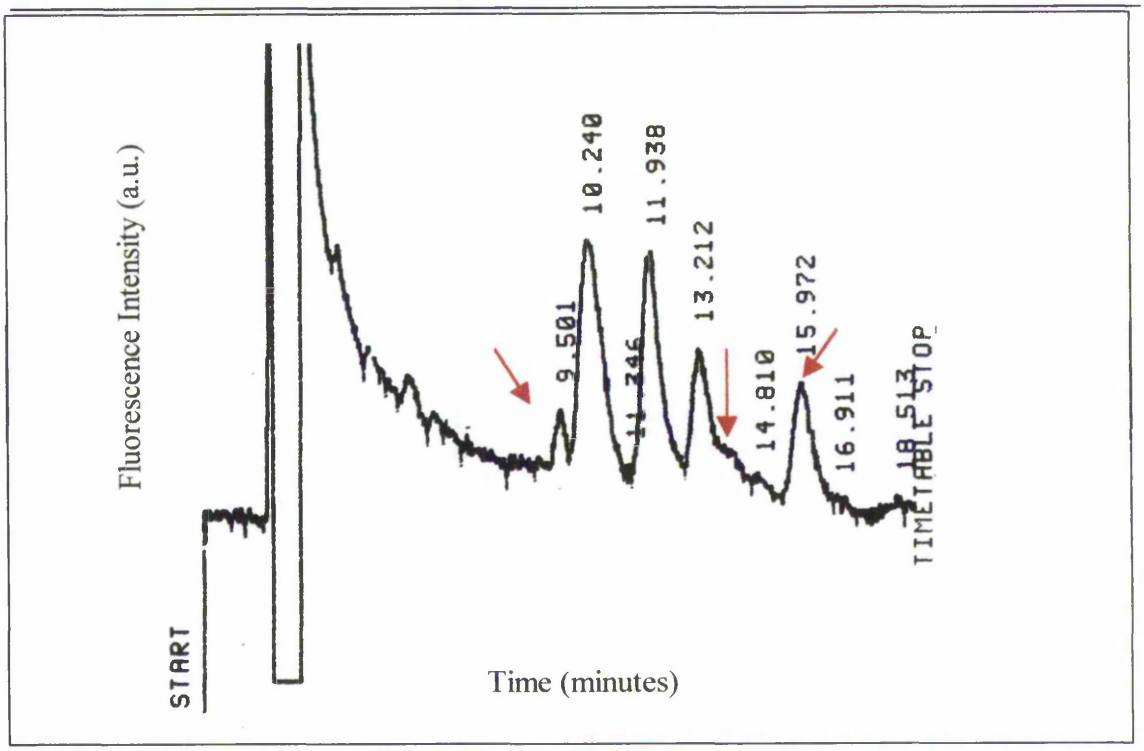

Figure 4.5 *E34 August survey chromatogram of a sediment extract, nonylphenol- 10.24, octylphenol- 11.938, butylphenol 13.212. The arrows indicate interference peaks.*

The sediment extracts were analysed in sets of 4 vials with a standard run before each set, all vials were analysed in triplicate using the method in Section 2.2.5. A standard sediment extraction chromatogram is shown using E34 August as an example (Fig. 4.5).

A number of unknown peaks were observed, with a majority of them causing no interference. For some sites, however, there was an interference observed as a shoulder on the butylphenol peak, this made integration difficult and potentially compromised the use of butylphenol as an internal standard. An external calibration method was employed for all samples rather than the use of an internal standard method as a result of integration problems. The analyte concentrations in the sediment samples were calculated using Eq. 2.12 and shown in Fig. 4.6 for May and Fig. 4.7 for August.

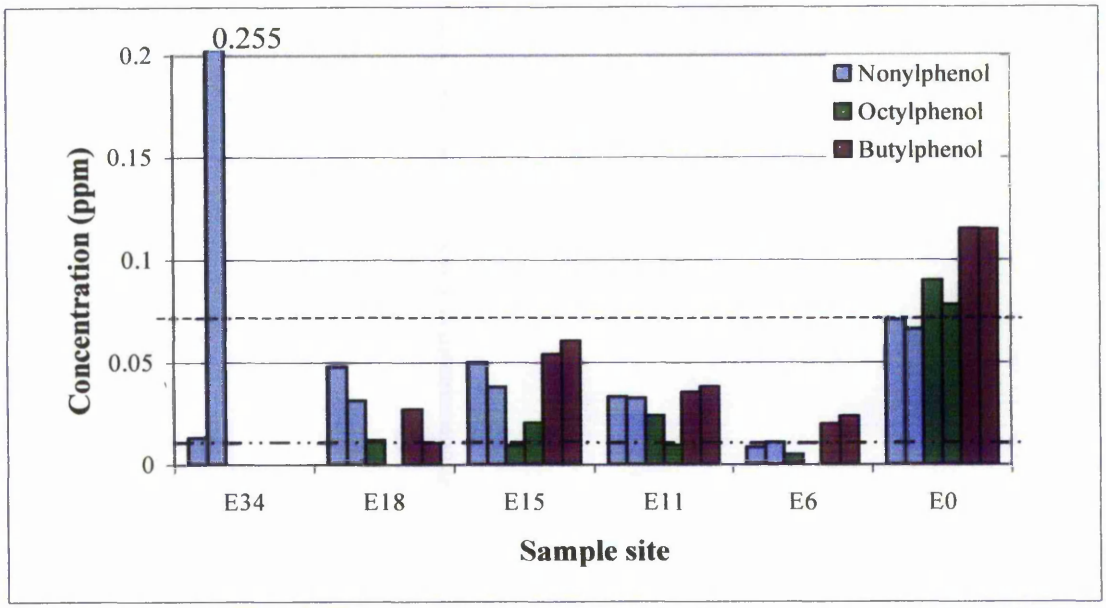

Figure 4.6 *Environmental concentrations of the 3 alkylphenols in the May survey. The (----) line indicates the expected concentration of the internal standard (butylphenol), the (—·-) line indicates the limit of detection of the method.*

For extracts from site E0-E18 there was good agreement between the duplicate SPE extracts for all analytes. Results for E34 do not follow this trend as the two values differ by a factor of greater than 10; the results cannot be statistically manipulated as there are only two data points. By considering the concentrations and the trends at the other sample sites, it would appear that the 0.255 ppm concentration was the erroneous result. The main factor that has lead to the conclusion was that all three analytes were present at similar concentrations (for the other sites), therefore if the highest result was true then you would expect comparable concentrations of octyl- and butylphenol.

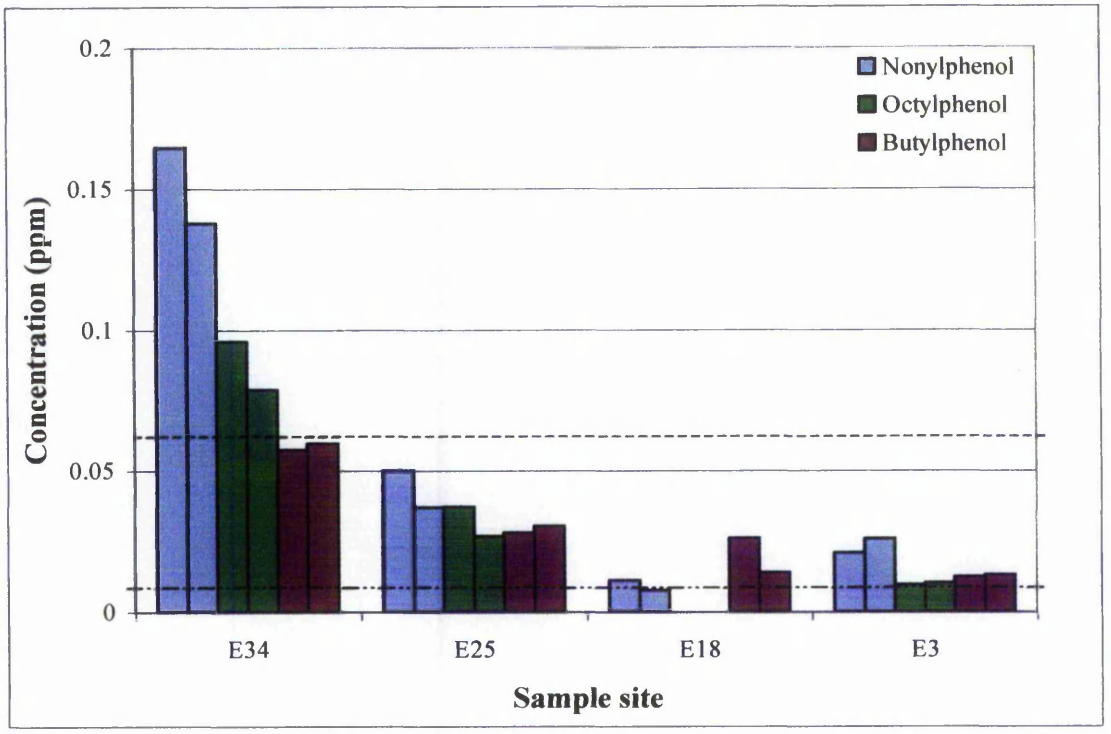

Figure 4.7 *Environmental concentrations of the 3 alkyphenols in the August survey. The (---) line indicates the expected concentration of the internal standard (butylphenol), the (—·)· line indicates the limit of detection of the method.*

The results in Fig. 4.7 again show good repeatability in the SPE method as also observed during the validation work on the standard solutions. The butylphenol recoveries are at least half that expected for sites 25, 18 and 3, this may be the result of integration problems with the interference peak or there may have been strong adsorption to the sediment as all analytes indicate a similar concentration. With no two sediments being of identical composition this added another variation factor for consideration. A number of the environmental concentrations were below the detection limit of the method (as defined by the blank plus 2 SD), they were included merely to demonstrate that their detection was possible and in a majority of cases the results were repeatable.

4.3.2.1 Trends associated with the environmental concentrations

The major constituents of sediment are organic and mineral material, the organic component is made up of decaying plant and animal matter and humic compounds, whereas the mineral component is mainly weathered rock, clay minerals and hydrous oxides. Pollutant adsorption onto the sediment is dependent upon the adsorption complex formed between the humic compounds, clay minerals and the hydrous oxides [Alloway and Ayres, 1997]. A relationship between carbon content of the sediment and its adsorption ability has been indicated [Neilson, 1994, Urano *et al.*, 1984]. From the results in this study (Fig. 4.8) such a trend is not immediately obvious and this would agree with a study carried out by Khim *et al.*, 1999b.

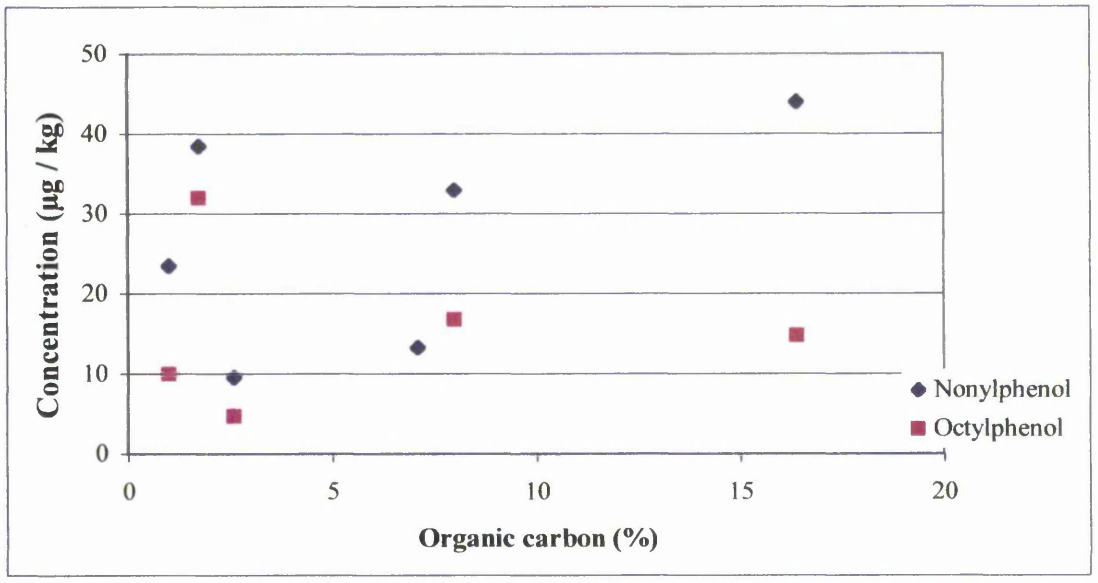

Figure 4.8 *Scatter plot of organic carbon content (%) and nonyl- and octylphenol concentration in sediment samples (µg / kg).*

Salinity is also thought to have an effect on the adsorption / desorption of organic pollutants, a study by Brannon *et al.*, 1991 suggests that there is an increase in desorption of PCB congeners occurring in distilled water over saline. The August sediments (Fig. 4.9) observe this trend (though the correlation coefficient values are far from ideal) with a greater concentration of the alkylphenols being observed in the sediment at higher salinity.

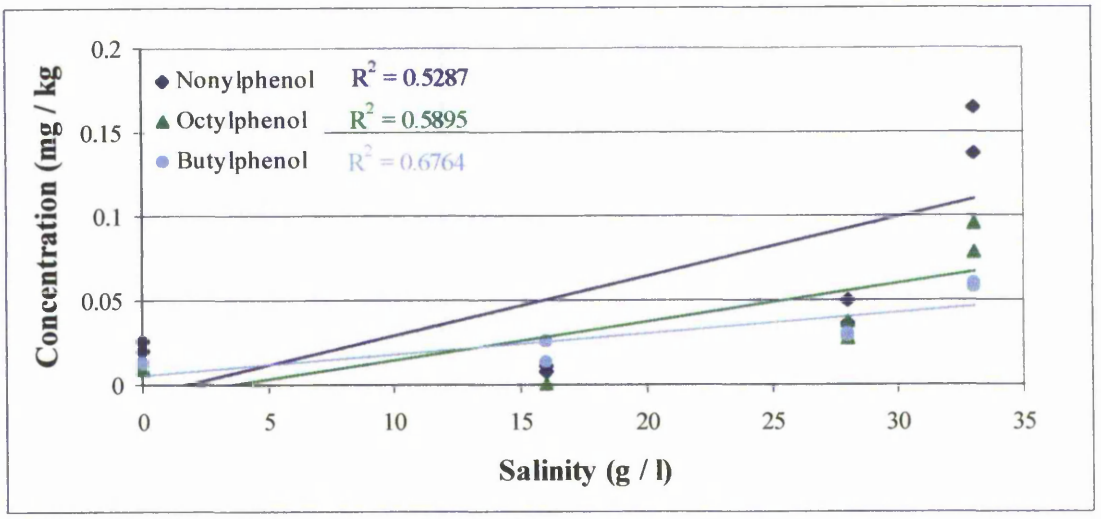

Figure 4.9 *Relationship between alkylphenol sediment concentration and salinity for the August survey.*

Such a relationship did not occur with the May survey (Fig. 4.10), in fact the reverse may be the case, with small correlation coefficient values suggesting that there is little confidence in the trend. This trend is possibly explained by work carried out by Podoll *et al.*, 1988, they noted that single analyte adsorption / desorption experiments were not giving a true picture of interaction and the presence of additional pollutants may increase or decrease bonding to the sediment. Podoll found that if poly (N,N – dimethylaminoethyl methacrylate) was adsorbed onto the sediment then there was a significant increase in the adsorption of naphthalene.

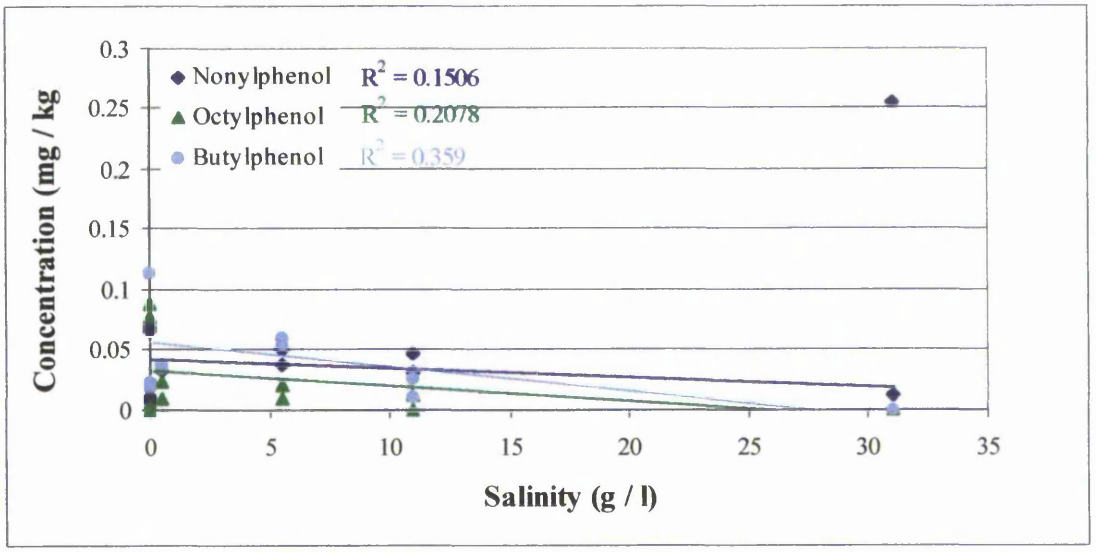

Figure 4.10 *Relationship between alkylphenol sediment concentration and salinity for the May survey.*

Another factor worthy of consideration is that of temperature, that results from the two surveys were pooled and displayed in Fig. 4.11.

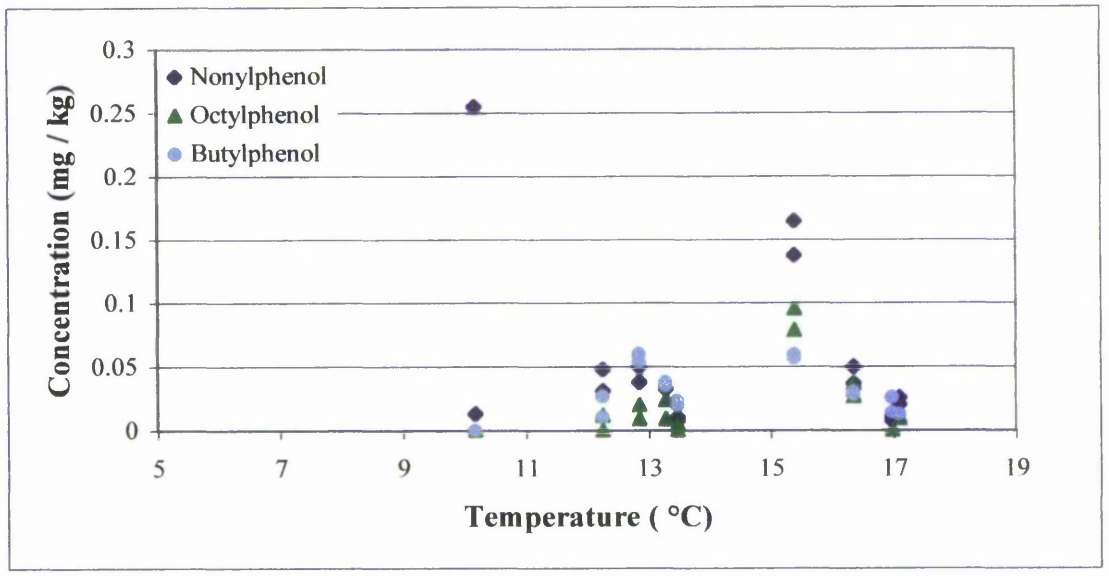

Figure 4.11 *Distribution of sediment concentration versus water temperature.*

The main conclusion that can be drawn from Fig. 4.11 is that an increase in temperature appears to give an increase in sediment concentration up to approximately 15 °C, if a linear regression line is generated for water temperature from 0 – 15.4 °C then the correlation coefficients for the three analytes range from 0.5325 – 0.6227. Above 15.4 °C there is a decrease in concentration with a linear fit producing correlation coefficients between 0.9483 – 0.9953. One possible explanation would be biodegradation, the conclusions drawn from Section 1.3.2 would suggest that if any biodegradation were to occur then it would be most likely to be at temperatures above 15 °C. Another explanation for the decrease above 15 °C would be the increase in water solubility of the compounds of interest, this would result in an increase in water concentration.

The decrease in alkylphenol concentration in sediment with temperatures below 15 °C cannot be easily explained as it would appear to go against all of the proposed trends. However, if the lower temperature / concentration points are put into context in terms of situation in the estuary then it is possible to explain the results. The lower temperature waters are found in the open estuary. The estuary has lower temperatures as a result of mixing with the cooler seawater and concentrations are lower as a consequence of dilution.

4.4 River and estuarine suspended solid analysis

4.4.1 Sample pre-treatment

The suspended solids were collected by filtration of a 2 l water sample taken at a specific site along the estuary, as described in Chapter 3. The filter papers were stored in pre-rinsed glass sample bottles. Prior to extraction the filter papers were dried at 60 °C in an oven. Once dried the filter papers were subject to soxhlet extraction under the conditions given in Section 4.2.1, no internal standard was added to the May water samples prior to filtration though the August samples were spiked with butylphenol at a concentration of 1 μg / l in a 2 l sample. The soxhlet liquor was again subject to duplicate SPE as for the sediment samples, results are found in Section 4.4.2.

Concentrations for the suspended solids are calculated using an external calibration method. The response factor (Rf) is obtained using Eq. 2.11 and the unknown concentration of the alkylphenols in the suspended solids is calculated using Eq. 4.1.

$$\mu g / l = Rf \times unknown\ area \times concentration\ factor \quad (10) \qquad \textbf{Equation 4.1}$$

The concentration factor is determined using the process below, the concentration used in the calculation is that of the internal standard.

2 µg in 2 l	- concentration in the original volume of sample prior to filtration
2 µg in 100 ml	- concentration of internal standard in the soxhlet extract
0.5 µg in 25 ml	- concentration and volume subject to SPE
0.5 µg in 5 ml	- concentration and final volume resulting from SPE
0.1 µg in 1 ml	- the calculated concentration upon HPLC analysis

Therefore if the HPLC value is multiplied by the Rf value to give mg / l and multiplied by 20, the concentration in the original 2 l sample is determined or multiplying by 10 will result in the concentration of internal standard in 1 l.

4.4.2 Suspended particle results

Only a few sites were analysed for suspended solids, there were three sites for the May survey and four for the August. The results are shown in Fig. 4.12 and 4.13. The limit of detection for the extraction and analytical method was 0.08 µg / l (ppb).

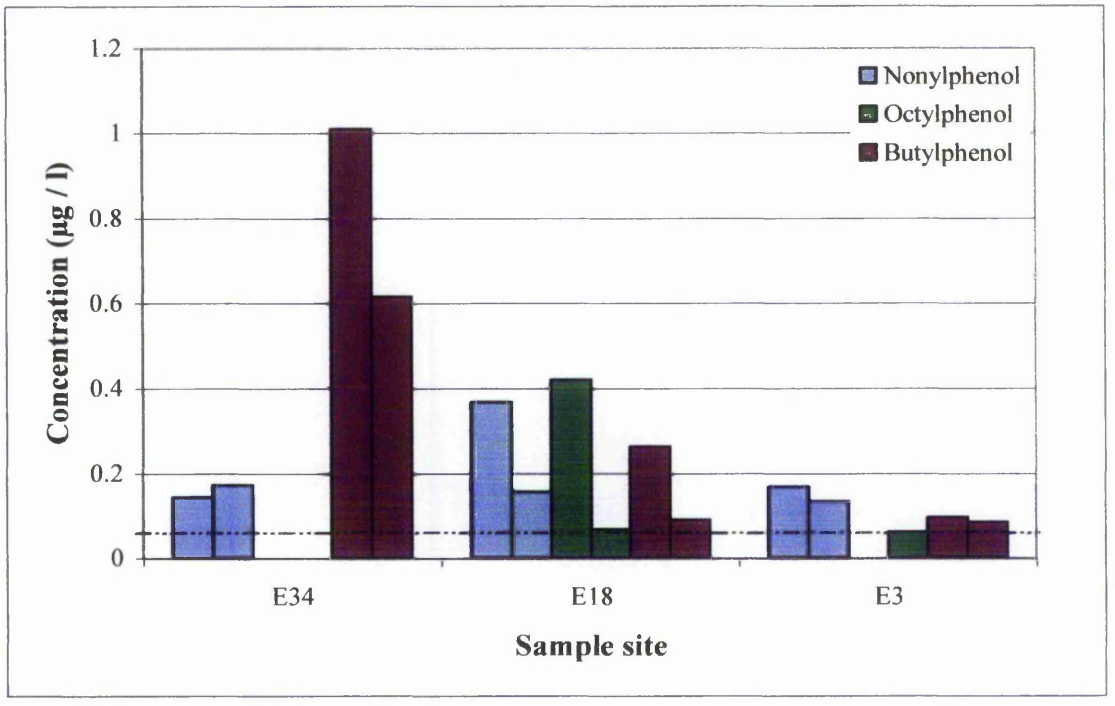

Figure 4.12 *May suspended solid alkylphenol concentrations, (—··) indicates the limit of detection.*

The nonylphenol environmental concentrations generally range from 0.17 – 0.13 μg / l, though with one exceptional value at 0.37 μg / l for one replicate of the E18 results. The even distribution throughout the estuary was a little surprising as point discharges would be expected to exhibit more variation within the estuary. Octylphenol would appear to be present to a lesser extent in the samples analysed, of the concentrations that were found 2 of the 3 were on the limit of detection for the combined method. The butylphenol was not added to any of these samples and yet it is present to a greater or lesser extent in all sites sampled. A number of possibilities can be drawn from these results:

- The samples were being contaminated in the lab during extraction, (though the source is inconsistent).
- Butylphenol was present in the environmental samples and therefore being used (and discharged) by industry.
- An interference was present in the estuary and appears on the chromatogram in virtually the same area as butylphenol.

The repeatability of the results was poor, indicating both systematic (E18) and random error (butylphenol, E34). One way to improve on these results would be to run each extract in triplicate rather than duplicate, confidence in the results could also be improved by the soxhlet extraction of a second sample from the same site.

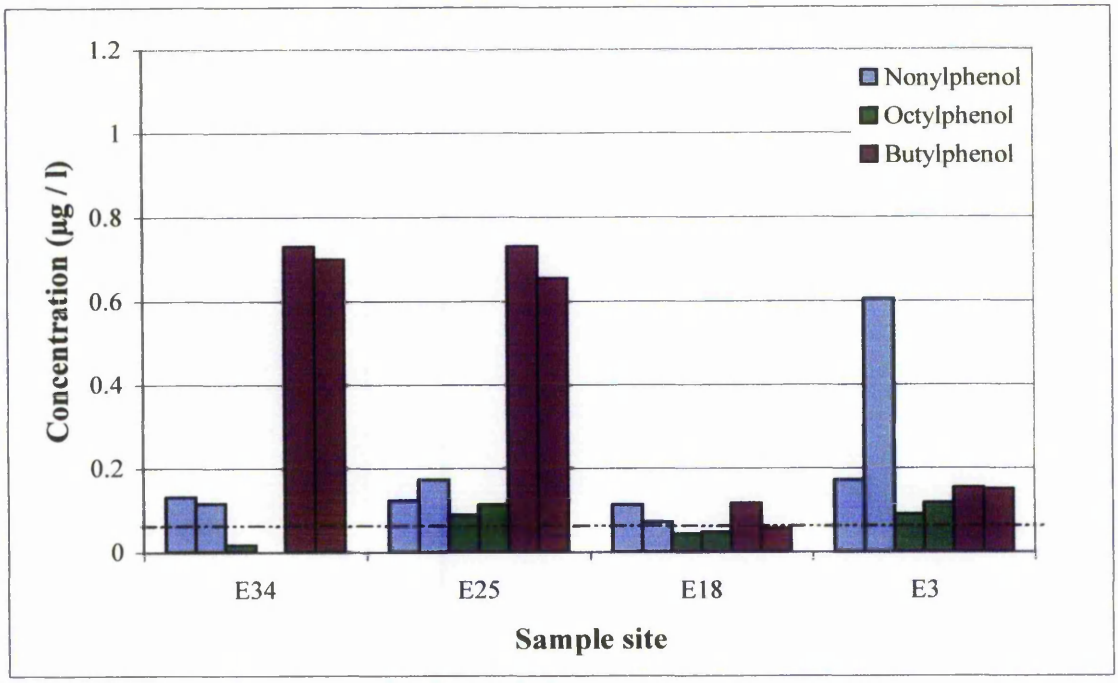

Figure 4.13 *Alkylphenol concentrations in suspended solids collected along the Forth Estuary in August 2000. The water sample prior to filtration was spiked at a concentration of 1 µg / l, the (_...) indicates the limit of detection.*

For a majority of the analytes of interest there was an even distribution along the estuary as with the May survey, with concentrations below 0.2 µg / l. The August concentrations have greater repeatability than the May results except for the second nonylphenol extraction for the E3 sample site. The most likely cause of this inflated concentration is contamination, though the source is unclear.

The environmental concentrations of nonylphenol are within a similar range to those observed in the May survey with values between < 0.08 – 0.17 µg / l, with a high concentration of 0.60 µg / l that are probably a result of contamination. The octylphenol values were lower than nonylphenol with 3 of the 7 results being below the limit of detection with the remainder of the values ranging from 0.09 – 0.12 µg / l. The butylphenol values for E34 and E25 were exceptionally high and though the water had been spiked prior to filtration the values were not consistent with those found in the water samples, where recoveries were around 70 % for the internal standard.

The most likely conclusion would be that there was a discharge point in the region of E34 and E25 that was either depositing butylphenol (which seems unlikely as its use in industry has not been reported) or a compound similar into the estuary. This conclusion would agree with the May survey where concentrations around 1 μg / l were determined for site E34. One possible candidate would be methylphenol as it would have similar retention characteristics to that of butylphenol and would probably fluoresce under the same wavelengths. Potential methylphenol sources include, coal tar and petroleum refining and dye, perfume and herbicide manufacture.

A relationship between analyte concentration and turbidity was expected as a result of the affinity of the alkylphenols to particulate matter. The trend expected was an increase in concentration with an increase in turbidity, with results shown in Fig. 4.14.

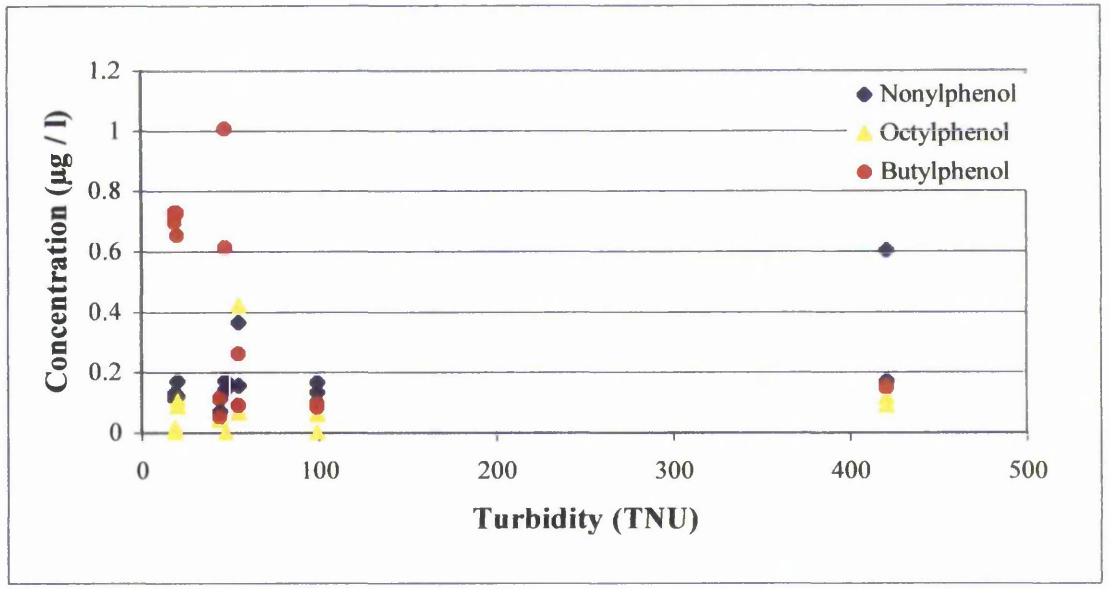

Figure 4.14 *Environmental concentrations of the three analytes versus turbidity.*

Butylphenol indicated a possible trend in opposition to that expected though the correlation value was only 0.1966. As a result of the small data set the results were inconclusive in terms of a relationship between turbidity and alkylphenol concentration, one explanation may be that other factors need to be taken into consideration such as, salinity and temperature.

4.5 Summary

The analytical and solid phase extraction methods have been validated with encouraging results produced. The soxhlet method however, was not validated as a result of no suitable reference material being available, this presents a potential source of error that is discussed in Chapter 5. The sediment and suspended solid results were again subject to an external calibration method as a consequence of the continued presence of an interference peak with a similar retention time of butylphenol.

The concentrations for the sediments were in the range of < 10.2 – 152 μg / kg for nonylphenol and < 10.2 – 87.4 μg / kg for octylphenol. These levels are comparable to those displayed in Table 4.1, though considerably lower than concentrations found in the Great Lakes of Canada and the US [Bennett and Metcalfe, 1998] and Tokyo Bay [Yamashita *et al.*, 2000]. This would possibly indicate that the Forth Estuary, whilst subject to alkylphenol pollution was not the result of gross contamination and high level input.

The suspended solid concentrations were detected in the range of < 0.08 – 0.26 μg / l for nonylphenol and < 0.08 – 0.26 μg / l for octylphenol. These results are not comparable to the results shown in Table 4.2 as a consequence of a difference in units. The results by Sekela *et al.*,1999, for Canadian rivers have been converted into ng / l but the data are at least two orders of magnitude lower than the concentrations detected in this study. This implies that the Forth may have elevated alkylphenol concentrations associated with suspended solids relative to a 'pristine' environment, and may be indicative of the presence of local alkylphenol inputs.

The concentrations of both sediment and suspended solids in the Forth Estuary are of significance, though levels are not critical. Sediment concentrations potentially pose a greater threat to aquatic organisms in comparison to the concentrations found in the water column. A majority of the estuary dwelling organisms may therefore remain unaffected by these concentrations, however, those that are sediment dwelling or filter feeders are at greater risk of alkylphenol contamination and bioaccumulation as confirmed by a study undertaken by Wahlberg *et al.*, (1990). The common mussel is one such organism that falls into the class of filter feeder and with bioaccumulation predicted at 3400 [Ekelund *et al.*, 1990] and LC 50 values of 0.14 mg / l determined for 850 hours [Granmo *et al.*, 1989], there is a potential situation of chronic mussel toxicity.

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Chapter 5: Conclusions and Further Work

5.1 Introduction

Although summaries have been drawn at the end of each of the results chapters it is important to look at the estuary system as a whole if alkylphenol distribution is to be put into context. This chapter will consider the alkylphenol concentrations found in the various matrices examined in this study and conclusions will be drawn from these data. From these conclusions a plan of further work will be suggested in order to further validate these findings and improve on the database of information now available on the Forth Estuary with regard to certain alkylphenols.

5.1.1 Comparison of nonylphenol and octylphenol concentrations found in water, suspended solids and sediment

The nonylphenol concentrations detected in the water, suspended solids and sediment are collated in Table 5.1, using the data from both the May and August surveys. Nonylphenol concentrations in water for the May survey were below the limit of detection but concentrations in the August survey ranged from < 0.15 – 1.9 μg / l. There is no immediate relationship between concentration and position along the estuary. The high concentrations appear at random points along the Forth and are likely to be a consequence of specific discharge sites. These levels are quickly dispersed and concentrations have decreased by the next sampling site.

The suspended solid results for both the May and August survey indicate only a small variation (apart from May E18) and this seems a little unusual considering the dynamic nature of an estuary system. There are a few possible explanations:

- The extraction method was not optimised for the suspended solid work and may therefore present a source of error.
- Degradation of the samples may have occurred as a result of the sample storage procedure.

However, the data may be accurate, as a result of the small sample data set it is not possible to draw firm conclusions as to which one of these explanations may be true. Given the anticipated analytical uncertainty of less than 20 %, unless sample integrity has been compromised, the nonyl- and octylphenol concentrations in the suspended solids would appear to be approximately 0.1 – 0.2 µg / l throughout the mixing zone.

Table 5.1 *Nonylphenol distribution within the water, suspended solids and sediment samples for the May and August 2000 surveys.*

	May			August		
Sample site	**Water (µg / l)**	**Suspended solids (µg/l)**	**Sediment (µg / kg)**	**Water (µg / l)**	**Suspended solids (µg/l)**	**Sediment (µg / kg)**
E42		nd	nd	bld	nd	nd
E34		0.16	13.3	bld	0.12	152
E25		nd	nd	0.17	0.15	38.5
E20		nd	nd	0.7	nd	nd
E18		0.26	40	1.9	0.09	bld
E15		nd	44	0.31	nd	nd
E13		nd	nd	0.79	nd	nd
E11		nd	33	0.14	nd	nd
E6	Below limit of detection	nd	bld	1.10	nd	nd
E3		0.15	nd	0.33	0.17	23.5
E0		nd	68.5	1.13	nd	nd

bld below limit of detection

nd not determined

The sediment data shows a certain amount of variation between each site, which is expected. The surprising result comes when the data from the May and August surveys from the same sample sites are compared. The results would be expected to indicate variation between sample sites but a certain similarity at the same site over time. This is not the case in this study, although only two sites were analysed from both surveys, the results are very different, varying from 13.3 to 152 μg / kg for E34 and from 40 to < 10.2 μg / kg for E18. As with the suspended solids it is difficult to draw conclusions without viewing a larger data set, there is again the possibility of extraction error but this can only be ruled out by further work. However, the calculated analytical error for the extraction of nonyl- and octylphenol from a solid matrix was between 1.6 and 59.8 % (typically < 20 %) over a concentration range of 0.001 – 0.5 mg / kg. These results would suggest that the variation in concentrations determined for the Forth Estuary are not purely the result of experimental error but actually represent the diversity of the sediment matrix and the problems associated with the determination of pollutants within the system. Similar variability has been observed in the analysis of metals within sediments, lead concentrations determined for 2 samples taken from the same site varied from 1.9 to 4.5 mg / kg as reported by Rheinallt *et al.*, (date not known).

The octylphenol concentrations determined from the two surveys, water, suspended solids and sediment are compared in Table 5.2.

Table 5.2 *Octylphenol distribution within the water, suspended solids and sediment samples for the May and August 2000 surveys.*

	May			August		
Sample site	**Water (µg / l)**	**Suspended solids (µg/l)**	**Sediment (µg / kg)**	**Water (µg / l)**	**Suspended solids (µg/l)**	**Sediment (µg / kg)**
E42		nd	nd	bld	nd	nd
E34		bld	bld	bld	bld	87.4
E25		nd	nd	bld	0.10	32
E20		nd	nd	0.39	nd	nd
E18		0.26	12.1	1.28	bld	bld
E15		nd	14.8	0.18	nd	nd
E13		nd	nd	0.18	nd	nd
E11		nd	16.8	0.33	nd	nd
E6	*Below limit of detection*	nd	bld	0.96	nd	nd
E3		bld	nd	0.17	0.10	bld
E0		nd	83.8	0.40	nd	nd

bld below limit of detection
nd not determined

Octylphenol concentrations in water are similar to those observed for nonylphenol with concentrations ranging from < 0.15 – 1.28 µg / l for the August survey but concentrations below 0.15 µg / l for May. E18 would appear to be the site of alkyphenol discharge with high concentrations for both nonyl- and octylphenol. From the map (Fig. 3.6) there is no obvious industry or sewage treatment works though the site of the Alloa sewage treatment works discharge is unclear.

The suspended solid data for octylphenol show little variation and are therefore comparable to those of nonylphenol, with concentrations in the range of < 0.08 – 0.26 µg / l. These results could indicate that the suspended solids are in a state of equilibrium in the environment and therefore variation in concentration is kept to a minimum. As mentioned previously, theories can only be proposed for the results from this work and further work will be required to confirm or deny these possibilities.

A comparison of sediment data is again possible for the two surveys at sites E34 and E18. Concentrations were in the range of < 10.2 - 87.4 μg / kg for E34 and < 10.2 – 12.1 μg / kg for E18. These concentrations are lower than nonylphenol, as would be expected, though still demonstrating the variation in concentration at the same site over time. A possible explanation for these results is dredging, this process may disrupt the sampling sites and would in effect alter the sediment concentration profile, this would require further investigation. For the sediment dataset in the Forth Estuary as a whole, nonylphenol concentrations ranged between < 10.2 – 152 μg / kg, whilst octylphenol ranged between < 10.2 – 87.4 μg / kg

5.1.2 Conclusions

The general trends observed would suggest that the greatest portion of both nonyl and octylphenol are found in the sediment, with a minority of the alkylphenol budget existing in the dissolved or suspended form in the water column. Analysed sediments revealed a concentration range of < 10.2 – 152 μg / kg for nonylphenol and < 10.2– 87.4 μg / kg for octylphenol. The highest concentrations of both nonyl and octylphenol are found in E0 for the May survey and E34 for the August survey. The high levels at E34 can be explained by the petrochemical industries and sewage treatment works that are based along the estuary banks but the concentrations at E0 are a little harder to explain. There is no heavy industry based along the banks of the river at E0, though it is subject to discharge from sewage treatment works, also as a result of the rural location, pesticide run off and wool scouring cannot be excluded as possible sources of alkylphenol pollution.

From Tables 5.1 and 5.2 there would appear to be a link between nonyl- and octylphenol concentrations in water and suspended solids. Concentrations between the matrices appear to be similar as would be expected if the suspended solids were not associated with effluent or industrial discharge. These results however, do not correspond to those found in the literature [Sekela *et al.*, 1999, Kvestak *et al.*, 1994], with water concentrations higher than suspended solids, sometimes by an order of magnitude. This could be the result of specific estuary effects where alkylphenol discharge is associated with the aqueous phase and as a result of water volume or rapid flow, the alkylphenols are not able to associate with the suspended solid matter in the same way.

The most important factor to be produced from this work is the evidence of alkylphenol presence in the Forth Estuary. The concentrations determined in the Forth Estuary are consistent with those associated with a contaminated, though not grossly polluted estuary system. The concentrations of nonylphenol and octylphenol detected were not at levels that would cause lethal toxicity to aquatic organisms, they were within the range to induce chronic rather than acute toxicity however if bioaccumulation was considered. Limited biomonitoring in the Forth catchment supports this contention, where Moore and Stevenson, (1994) found intersex in benthic copepods, it may be that nonylphenol and octylphenol contribute to this effect. Further investigations into distribution and source of the alkylphenols are vital in order to build up the database of information available on this estuary. Biological studies would also be interesting with regard to fish exposure and the possibility of intersex and vitellogenin production.

5.2 Further work

There will always be improvements that can be made with any analytical method, whether it is enhanced sensitivity or greater sample concentration. This section will consider the problems that were encountered during this study and suggest possible options on how these problems may be overcome.

5.2.1 Water analysis

The water analysis method was shown to be a robust procedure that produced both repeatable and reproducible results. However, as a consequence of the low environmental concentrations, improvements on the limit of detection would be an advantage. Improvements may be achieved by increasing the concentration step, this can be accomplished by increasing the sample volume to be extracted or reducing the volume of extract post-solid phase extraction. Problems may be encountered whichever route is chosen, an increase in sample volume will possibly result in analyte breakthrough, though this can be offset by using a larger SPE cartridge containing more packing material. Reducing the extract volume is possible by drying down under nitrogen, problems can arise from analyte adsorption to the glass vial, leading to a loss in recovery.

Another area of the water analysis that could benefit from further work would be that of sample preservation. The use of mercury (II) chloride was possibly not the best preservative as there are a number of safer alternatives such as, formaldehyde or chloroform [Lee, 1999]. Investigation into these alternative preservatives would be useful, in terms of quantity needed for the prevention of biodegradation and the length of time that the preservative would be effective over. Other considerations when choosing a preservative are the effects it may have on the extraction or analytical method, the preservative compound may have similar retention properties to the analytes of interest or the same wavelengths of detection.

With all three matrices there was a problem with contamination and it would be worth investigating this problem further. The identification of the interference and its source whether from the sampling site or the laboratory would be of interest if further work were to be pursued.

5.2.2 Sediment and suspended solid analysis

The main problem with the sediment analysis was the validation of the soxhlet method without the use of a standard reference material, this problem may be overcome by producing a reference material. This may be achieved by homogenising a bulk sediment sample and exhaustively extracting over a 24 hour period with a suitable solvent. The surrogate reference material may then be subject to validation by spiking at varying concentrations and recording the recovery results. Duplicate extractions could also be employed to determine reproducibility between extraction systems and repeatability within an extraction system.

The concentration step for the sediment extraction was adequate though some of the sample concentrations did fall below the limit of detection. There are two possible ways to improve on the limit of detection:

- Increase sample size for soxhlet extraction
- Increase volume of extract subject to solid phase extraction

To increase the sediment sample size larger soxhlet apparatus would be required and hence larger solvent volumes, this would therefore result in only a small, if any, increase in concentration. Increasing SPE sample volume is possibly a better option, as it is easier to validate and has the potential of a four-fold increase in concentration.

5.2.3 Suspended solid analysis

Validation of the soxhlet method for this matrix is required and could possibly be achieved by the spiking of solvent washed filter papers. The method would not be able to account for the effect of the particulate matter on the extraction procedure but it may be possible to draw conclusions from the sediment validation work to compensate. The other problem with the suspended solid work was sample storage. Investigations into this area would be advantageous with several options available;

- Use of preservative on the wet filter paper prior to storage.
- Oven drying the filter paper
- Freeze the filter paper to prevent degradation.

5.2.4 Fish tissue analysis

One area that was not looked at in this study but would be worth investigating is concentrations of nonylphenol and octylphenol in fish and other aquatic organisms. Several papers have suggested possible methods, the most interesting being that of matrix solid phase dispersion extraction (MSPD) [Zhao *et al.*, 1999]. This method combines extraction, concentration and clean up into one step, a biological sample is homogenised with C_{18} SPE material before being packed into a glass column over a layer of alumina, the analytes are then eluted with an appropriate solvent. Depending on the success of this method in the analysis of biological samples it may then be possible to apply to sediment extraction.

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