

the identification of oil samples in large volumes of aqueous media e.g. an oil slick in the sea, is that any microtrace or identifier introduced into oil has a tendency to partition out or be dispersed in the aqueous phase rendering collection and identification of the microtrace particularly difficult.

Many tracing methods have been used to solve problems of this sort, all involving the addition of some characteristic material. Previously such additives have been dyes, or radioactive compounds, or characteristic organic molecules, or cellulose microdots. Biological materials have also been used, notably for tracing the movement of water bodies in the natural environment, such as bacteriophage or bacteria. In these cases the living systems possess some property (for example, a known drug resistance pattern, or particular host specificity) which does not normally occur in nature. The added organisms can be traced from their point of addition by measuring samples as required, isolating the added organisms and showing that organisms isolated from samples is the same as that originally added.

International Patent Publication No. WO 90/14441 published on 29th November, 1990 discloses a method of monitoring the presence of a substance which comprises tagging the substance with a nucleic acid, collecting the substance and detecting said nucleic acid, generally by amplifying the nucleic acid using polymerase chain reaction technology. Suitable substances which may be tagged are said to include air pollutants, oils, aromatic compounds, explosive compositions, foodstuffs, medicaments, inks, paper goods and paint products. It is stated the nucleic acids can be optionally bound to a component of the tagged substance through a covalent bond i.e. that the nucleic acids are covalently bound to the tagged material or a component thereof. Alternatively, the nucleic acids may be free or they may be bound to a solid support (such as latex beads, dextran or magnetic beads) which is then mixed with the material being tagged.

International Patent Publication No. WO 90/14441 discloses that oils and other non-polar liquids can be tagged effectively by the use of detergents added to the taggant prior to the addition of the taggant to the liquid. Recovery of taggant may be achieved by standard techniques. Typically, the sample is washed or extracted with either distilled water or a buffered solution. Using phenol based extractions or phenol/chloroform extractions one can recover nucleic acid from complex biological substances or from oil based substances.

International Patent Publication No. WO 90/14441 does not appreciate the problem of tagging petroleum products in a manner which will withstand prolonged mixing with vast amounts of aqueous phase nor disclose the possibility of tagging immense volumes of oil e.g. thousands of tonnes, which are currently routinely carried over the seas by tankers. The specific example of tagging oil comprises:

Preparation: (a) mix together 40 μ g of taggant, 10 μ l "Tween[®] 80" (detergent), 10 μ l "Span 80" (detergent), and 100 μ l distilled water, and (b) add mixture to 1.7ml oil. The combination is then thoroughly mixed.

Recovery: add oil directly to PCR mix (which is an aqueous based mixture), vortex and amplify; or use a standard phenol-chloroform extraction. After treatment with phenol, the taggant was detected in the boundary layer between the oil and water phases.

WO87/06383 describes methods of labelling materials using a predetermined macromolecular first compound and detecting the first compound using a complementary second compound which binds to the first compound. Genomic DNA and DNA from a partial restriction endonuclease digest thereof are exemplified.

This invention describes the use of a particular biological material which can be used to monitor the movement of materials in any of the general ways envisaged above. The biological material used is a complex molecule whose structure can be characterised in several ways and in principle an infinite number of unique molecules can be generated to produce an infinite number of different, unique microtrace additives. In practise, the natural variability of the molecule can be manipulated to give a measurable value for the likelihood of such a similar molecule occurring naturally. That is, by careful selection of molecules for the microtrace

material complete certainty within prescribed limits of probability can be calculated to show that there can be only one outcome of a particular trace pattern. The present intervention makes use of recent advances in highly sensitive methods for the detection of the presence of this biological molecule at exceedingly low levels or concentrations. Moreover this procedure, although based on a biological molecule, does not depend on the system being living within the normal definition of living, i.e. capable of sustained replication from one generation to the next.

The biological molecule to be used as the microtrace additive is DNA (deoxyribose nucleic acid), the genetic information molecule of almost all living systems. Each DNA molecule can be unique as a result of the sequence of bases (adenine, thymine, cytosine and guanine) contained within the molecule. Probability terms can be calculated for the frequency of a given sequence of bases and, so long as sufficient bases are used (i.e. a sufficiently big DNA molecule is employed as the microtrace additive) then for all practical purposes a unique microtrace can be defined and used. Previously DNA (or other biological molecules) could have been used as trace molecules, but the sensitivity of all known analytical procedures precluded their use since large quantities of DNA would have been needed. Addition of such quantities as a trace may have been undesirable and moreover the process would have been uneconomic.

This invention describes the use of small quantities of DNA (typically in the concentration range 1000 to 0.01pg DNA/ μ l) because of the technical ability to determine the presence of DNA molecules made possible by the polymerase chain reaction method. In vitro methods have been described which allow for the enzymatic amplification of specific DNA sequences using oligonucleotide primers which recognise all or part of the DNA molecule used as the microtrace additive. The sequential use of the polymerase chain reaction enables the molecule to be amplified exponentially. For example,

amplification. However, this need not be the case: any DNA could be used and visualised using the following procedure.

DNA isolated from a sample following its addition as
5 a microtrace compound, and cleaved into small fragments
using any suitable restriction endonuclease (e.g.
EcoRI , PstI, HindIII, etc). To the digested or
partially digested, DNA microtrace isolated sample known
sequence double stranded linker molecules (typically of
10 15 to 25 bp long) added to the mixture and ligated to the
digested DNA molecules using DNA ligase enzymes. DNA
amplification of the DNA sample molecule could then be
achieved using single stranded DNA primers which
recognise the DNA linkers bonded to the microtrace DNA.

15 The uniqueness of the microtrace DNA molecule can be
pre-determined using specific DNA base sequences. The
DNA microtrace molecules may be ones which occur
naturally, such as pBR322, and for which a known sequence
has been determined (by DNA sequencing procedures). The
20 DNA used may be synthesised DNA using a pre-determined
sequence of bases DNA primers and linkers for unique
recognition which can be routinely synthesised. The
uniqueness of the microtrace DNA, primers and linkers is
known only to the individual who adds the microtrace
25 molecule, thereby guaranteeing security of microtrace
paths.

The DNA may be added directly as a naked molecule or
as part of a complete organism (phage, bacteria, fungus
or protozoa). The DNA may be formulated into materials
30 which protect the DNA whilst it is present in the
material being traced (e.g. as a bead surrounded by a
gelatin coat, or surrounded by another protective
polymer). DNA may be formulated in such a way as to
ensure that it dissolves or bonds to the material being
35 traced (e.g. for use in hydrocarbons it may be formulated
within a hydrocarbon soluble material which ensures the
DNA dissolves in the hydrocarbon and cannot be removed
easily by aqueous washing).

3. The region BC is the variable region of the microtrace DNA and it will be this region which gives each microtrace DNA molecule its unique, characteristic signal. If this region is 10 bases long then with the four bases available for a DNA molecule, there will be 1.048×10^6 unique microtrace molecules capable of being synthesised. If the BC region is 15 bases long, then 1.07×10^9 unique microtrace molecules can be synthesised. If the BC region is 30 bases, then 1.15×10^{18} unique microtrace molecules can be synthesised.

4. The region CD will be synthesised with methylated nucleosides to provide the hydrocarbon anchoring properties.

5. The biotin molecule for extracting by streptavidin binding will be attached to point A.

6. Thus, the model microtrace DNA may look like:

A	pa 30	B	10-30	C	ca30	D
18/20 <bases> for PCR primers	10 <bases> for DNA sequencing primers				methylated DNA with same structure as AB region	
aqueous phase					hydrocarbon phase	

AB and CD regions will be identical and constant for all microtrace molecules; except that the CD is methylated, AB is non-methylated and the biotin molecule is bound to point A.

In the case of DNA labelled with hydrophobic haptens, separation from a sample may be achieved by making use of hapten/antibody pairings in a similar manner to biotin/streptavidin as discussed above.

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In an alternative way to ensure that the DNA remains within the hydrocarbon, the microtrace DNA may be covalently linked to hydrophobic beads designed to be soluble in hydrocarbons and not in the aqueous phase. Such beads may comprise polystyrene or polyvinylchloride. Such beads generally have a size in the range 1 to 5 microns. If only a few oligonucleotides, but not enough for

1 A method of monitoring the movement of a hydrocarbon which comprises adding to the hydrocarbon, as a microtrace additive, DNA molecules to provide a concentration of microtrace additive in the range 0.01 to 1000pg DNA/ μ l, sampling the resulting hydrocarbon after movement thereof and detecting the presence of said microtrace additive in the sample, said DNA molecules being formulated as a hydrocarbon compatible material which ensures the DNA dissolves in the hydrocarbon and cannot be removed easily by aqueous washing.

2 A method as claimed in Claim 1 in which the microtrace additives dissolves or bonds to the hydrocarbon.

3 A method as claimed in Claim 1 or Claim 2 in which the DNA is formulated within a hydrocarbon soluble material which ensures the DNA dissolves in the hydrocarbon and cannot be removed easily by aqueous washing.

4 A method as claimed in any preceding claim in which the hydrocarbon is oil.

5 A method as claimed in any preceding claim in which at least a region of the DNA molecules are methylated.

6 A method as claimed in Claim 5 in which the DNA molecules are fully methylated.

7 A method according to any preceding claim in which a portion of the DNA molecule is linked to a biotin or a hydrophobic hapten.

8 A method as claimed in Claim 7 in which a portion of the DNA molecule is linked to a biotin and said microtrace additive is removed from the sample by contacting the sample with a solid phase coated with streptavidin or avidin.

9 A method as claimed in Claim 7 in which a portion of the DNA

molecule is linked to a hydrophobic hapten and the microtrace additive is removed from the sample by contacting the sample with a solid phase coated with an antibody for said hapten.

10 A method as claimed in Claim 7 or Claim 9 in which the hydrophobic hapten is selected from fluorescein, dinitrophenol and triiodothyronine.

11 A method as claimed in any of Claims 1 to 4 in which the microtrace additive comprises DNA incorporating sulphur nucleotides containing thiophosphates which are modified by suitable agents to render the molecule hydrophobic.

12 A method as claimed in any of Claims 1 to 4 in which the DNA is covalently linked to hydrophobic beads which are lipophilic and forms stable dispersions in liquid hydrocarbons.

13 A method as claimed in Claim 12 in which the hydrophobic beads comprise polystyrene or polyvinyl chloride.

14 A method as claimed in Claims 12 or 13 in which the hydrophobic beads are paramagnetic.

15 A method as claimed in Claims 12 to 14 in which the material is oil and the hydrophobic beads have a specific gravity substantially the same as that of the oil.