# Chapter 2 Literature Review

## 2.1 Introduction

This review outlines the properties of tracers and then focuses on the character of fluorescein as a tracer compound. The behaviour of fluorescein and its ionic forms are described as well as the methods used to measure its concentration and determine its ionisation constants. The principles of absorbance and fluorescence spectrophotometry are also reviewed, as these topics need to be integrated to provide a more complete understanding of fluorescein and its behaviour.

## 2.2 Tracers

Tracers are selected for their ability to be transported through a water system in a manner similar to the item of interest. A good tracer will be a stable substance both hydrochemically and microbiologically, and show no reactivity with the system components, e.g. sorption, ion exchange, or precipitation (Behrens, 1986). This reactivity depends on the tracer and system interaction and is not just a tracer characteristic, e.g. lithium ions behaved conservatively during a contact reactor test (Pretorius and Pretorius, 1999) but were not detectable after passage through a limestone aquifer (Atkinson *et al.*, 1973).

The ideal tracer will have reliably quantitative detection at low concentrations (Behrens, 1986). One recent tracer investigation reported its detection limits relative to the injected tracer concentration as between $5 \times 10^{-6}$ and $2 \times 10^{-5}$ (Meigs and Beauheim, 2001) for an organic tracer detection system based on high-performance liquid chromatography. If the same index is used an appropriate detection level limit appears to be about one millionth of the tracer injection concentration or better, with this level being achieved in the Atkinson *et al.* (1973) investigation for the fluorescent tracer pyranine, and the Pretorius and Pretorius (1999) investigation using lithium. Quantitative tracer detection is not just a tracer characteristic but also depends on the background noise of a system. This influence can be seen in the choice of iodide instead of bromide or chloride within brine solutions (Meigs and Beauheim, 2001), or in background fluorescence affecting the choice of fluorescent tracer (Smart and Laidlaw, 1977).

Tracers need to be stable because tracer mass recovery is viewed as confirmation of a properly conducted field trial and necessary to obtain accurate measurement (Feuerstein and Selleck, 1963). Tracer recovery is also important because conservative elution patterns can be tested using different mathematical models to quantify the flow character (Levenspiel, 1972) and the total amount of recovered tracer is used as a validity test for this approach.

Behrens (1986) challenges the concept of a single "ideal" tracer by comparing the movement of bromide ions with that of tritiated water. In his example bromide and tritiated water are simultaneously spiked into water entering a soil column. The bromide ions are confined to the free flowing water as they move through the soil column, because their negative charge excludes them from the negatively charged soil pores. Meanwhile, the tritiated water exchanges freely with all the water bound within the soil pores and moves more slowly. Therefore bromide ions and tritiated water measure different parameters and the tracer choice depends on whether the free water or the real water movement is more important to the investigator.

A tracer's safety, toxicity and persistence also need to be considered as is illustrated by Webster (1996) where radioactivity was detected in sampling wells at levels 18,000 times greater than the original background almost five years after the use of a tritiated water tracer.

There are thus several important criteria to consider when selecting a tracer, i.e. how well it measures the study system, is it conservative, can it be detected easily and quantitatively, and will it be safe to use? These considerations are based not just on the tracer character but also on its interaction with the system being measured, and the final choice may represent a compromise between the different selection criteria. Some compromise is implicit in tracer selection because apart from the radioactive isotopes of hydrogen and oxygen, all other tracers are compounds that have chemical and physical properties different from those of water (Behrens, 1986).

## 2.3 The role of tracers

Tracers have been used in a wide variety of water investigations. These include evaluations of the:

- Transport processes of subsurface flows by injecting tracer and then monitoring tracer concentrations in wells around the injection site (Behrens, Moser and Wildner, 1977, Hendry, Wassenaar and Kotzer, 2000, and Meigs and Beauheim, 2001).
- Contribution of cave waters to surrounding springs by adding different tracers to particular cave regions and monitoring their concentrations in the spring waters (Atkinson *et al.*, 1973).
- Effective porosity and dispersivity within an aquifer system (Carleton, Welty and Buxton, 1999).
- Solute transport processes and ground and surface water interactions of a river system (Paybins *et al.*, 1998) or its velocity and dispersion characteristics (Boning, 1974, and Lee, 1995) by releasing a plug of tracer and then monitoring tracer concentrations at different points downstream.
- Mixing regime of a treatment system (Levenspiel, 1972), such as chlorine contact tanks (Pretorius and Pretorius, 1999).
- Flushing effect in a distribution system (Malatesta and Schwartz, 1985).
- Stabilisation pond character (Shilton *et al.*, 2000)
- Dispersion patterns in anaerobic ponds (Peña, Mara and Sanchez, 2000)
- Parameters used for activated sludge modelling (Makinia and Wells, 2000)
- Flow patterns in strata around radioactive waste storage sites (Webster, 1996).
- Percolation of rainwater through soils (Reynolds, 1966).
- Solute transport mechanisms of rain through snow (Feng *et al.*, 2001).
- Suitability of tracers as indicators of pesticide transport (Sabatini and Austin, 1991).

Just as there is a wide variety of tracer applications there is a similarly wide variety of tracer types. Behrens (1986) listed a number of these including not just the fluorescent tracers such as Uranine (Fluorescein), Eosin, Pyranine, Sulforhodamine G and B, Rhodamine B and WT, Lissamine FF, and Napthionate, but also the other types such as the bromide ion, complex compounds such as rare-metal chelates, and the radioactive tracers such as tritiated water. The relative performance of these tracer types can be assessed in trials that test the same system with a variety of different tracers, e.g. lithium acetate, polyethylene powder,

lycopodium moss spores and pyranine (Atkinson *et al.*, 1973), or seventeen different chlorobenzoic and fluorobenzoic acids in combination with iodide (Meigs and Beauheim, 2001).

## 2.4 Fluorescein

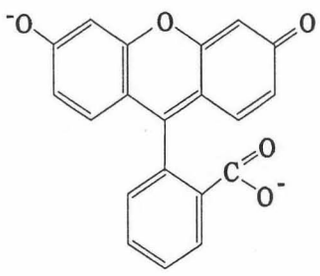
Fluorescein (CAS 2321-07-5) is a fluorescent organic compound with a chemical formula of $C_{20}H_{12}O_5$ (MW 332.3g). The chemical name of the free acid form of fluorescein is 3',6'-Dihydroxyspiro[isobenzofuran-1(3H),9'-[9H]-xanthen]-3-one (Dictionary of Organic Compounds, 1996) and its molecular form is often depicted as the lactone neutral species shown in Figure 1 (Zanker and Peter, 1958).

Other names for fluorescein include resorcinolphthalien, and Colour Index (C.I.) solvent yellow 94 (Dictionary of Organic Compounds, 1996), as well as D&C yellow no. 7 (Merck, 1989). The disodium salt of fluorescein (CAS number 518-47-8) has a molecular mass of 376.3g and also has a number of alternative names; fundusein and C.I. acid yellow 73 (Dictionary of Organic Compounds, 1996), disodium fluorescein, sodium fluorescein, soluble fluorescein, resorcinolphthalien sodium, uranine yellow, D&C yellow no.8, Ak-fluor, Fluorescite, Fluorets, Ful-glo and I-rescein (Merck, 1989). In some cases the terminology is not clear as the same chemical form has different names, as shown in Table 1.

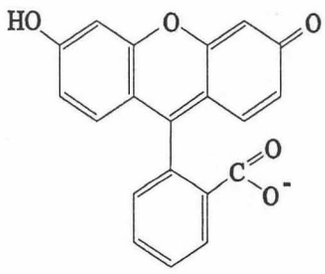
**Table 1 Examples of terminology conflicts**

| | Dictionary of Org. Compounds (1996) | Merck Index (1989) |
|---|---|---|
| Fluorescein (free acid) | Uranin | C.I. 45350 |
| Disodium fluorescein | C.I. 45350 | C.I. 45350, Uranin(e) |

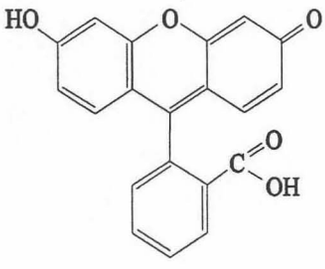
In this investigation both fluorescein and disodium fluorescein will be referred to as fluorescein. This choice of terminology is based on the assumption that the sodium atoms of disodium fluorescein dissociate immediately upon dissolution, leaving a fluorescein molecule indistinguishable from the free acid at the equivalent pH. This is contrary to the observation that the failure to distinguish between the free acid and disodium fluorescein has given rise to problems in the past (Markuszewski and Diehl, 1980), however these researchers are referring to the solid compounds in ether, and do not indicate any problem with the aqueous forms.



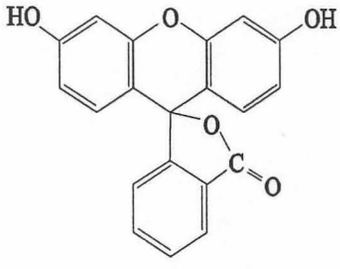
DIANION



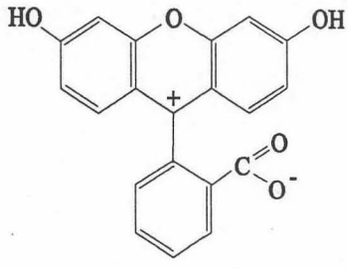
MONOANION



QUINONOID

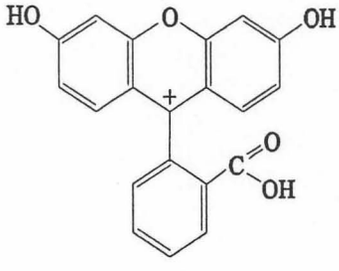


LACTONE



ZWITTERION

NEUTRAL SPECIES



CATION

**Figure 1 Six ionic forms of fluorescein**

## 2.5 Ionic forms of fluorescein

Dissolved fluorescein may be present as cation, neutral, anion, or dianion species and these ionic forms have all been associated with their characteristic pH ranges, from acid to alkalis respectively (Zanker and Peter, 1958). Three different species of neutral molecule have been distinguished; a quinonoid, lactone and zwitterion, and these are illustrated in Figure 1 (Zanker and Peter, 1958, and Martin and Lindqvist, 1975).

The characteristic fluorescence and absorbance spectra of the different ionic forms have been identified by comparing the fluorescein spectra with those of similar compounds, such as 6-hydroxy-9-phenyl-fluoron (Martin and Lindqvist, 1975) or monomethyl fluorescein, dimethyl fluorescein and fluorescein methyl ester (Chen *et al.*, 1979). These comparisons provided corroboration for the ionic forms suggested by Zanker and Peter (1958) and also refined Lindqvist's (1960) suggestion by proposing that the neutral form of fluorescein comprises about 5/8 lactone, 2/8 zwitterion and 1/8 quinonoid forms (Martin and Lindqvist, 1975).

Some researchers have suggested that fluorescein has just two ionisation species, a colourless leuco compound at low pH and a fluorescent "quinoid" that forms at a higher pH (Smart and Laidlaw, 1977). This conflicts with the data from other studies (Zanker and Peter, 1958, Lindqvist, 1960, Martin and Lindqvist, 1975, and Chen *et al.*, 1979) and cannot be supported as no evidence was provided to justify the two-species model. Furthermore the absence of an isosbestic point within the fluorescein spectra argues against the existence of just two forms because compounds such as pyranine that have two aqueous forms, have a pronounced isosbestic point (Launay *et al.*, 1980).

Klonis and Sawyer (1996) noted that while researchers had agreed on the different ionic forms the interchanges between these components had always been analysed using a single model. They proposed that as the different component spectra are agreed, it was possible to test different models of association and compare the fit. Klonis and Sawyer (1996) tested five different models and confirmed the cation, neutral, monoanion to dianion conversion sequence with increasing pH, but also suggested that the neutral species comprises 70 % lactone, 15 % zwitterion and 15% quinonoid. This is similar to the 62.5 % lactone, 25 % zwitterion and 12.5 % quinonoid prediction of Martin and Lindqvist (1975).

## 2.6 Advantages of fluorescein as a tracer

The three main advantages of fluorescein are that it is easy to detect, has a fluorescence response that is relatively temperature insensitive, and it is comparatively resistant to adsorption.

### 2.6.1 Easy to detect

The most important characteristic of fluorescein is its ease of detection. Minimum fluorescence detection levels of 0.000041 mg/l have been reported (Feuerstein and Selleck, 1963) in the absence of background fluorescence. Background fluorescence plays a crucial role in determining this level as the minimum detection level rose to 0.000058 mg/l in the same study when background fluorescence was present, and a level of 0.00029 mg/l was reported for a different study using the same fluorometer model in the presence of background (Smart and Laidlaw, 1977).

Fluorescein's fluorescence detection levels are typically orders of magnitude better than its absorbance detection levels. This can be illustrated by comparing the following absorbance example with the performance noted earlier for fluorescence. The absorbance of a compound can be calculated using the Beer-Lambert law, Absorbance = $\varepsilon bc$. If fluorescein's molar absorptivity ($\varepsilon$) is taken to be 89320 $M^{-1}$ $cm^{-1}$ (Heller *et al.*, 1974), and a one cm sample path length (b) is used, then a 1 mg/l solution of disodium fluorescein (molarity of about $2.66 \times 10^{-6}$ (c)) will give an absorbance of 0.24, which is equivalent to a transmission of about 58 %. If the spectrophotometer readout can distinguish between 0.1% transmission units then a concentration of 0.0017mg/l ($4.5 \times 10^{-9}$ M) fluorescein would be resolvable at low absorbance ranges. This example is only intended to provide a rough comparison because resolving ability and minimum detection levels are different parameters and it is not possible to predict actual minimum detection levels because these will be a function of instrument performance and sample background but this example does show that while fluorescence is a more sensitive method for measuring fluorescein concentrations the absorbance method is still a sensitive detection method.

If more modern detection methods are used, such as capillary electrophoresis in conjunction with laser-induced fluorescence, or preconcentration steps are taken, then detection levels can be extended down as far as $10^{-12}$ M (Ferguson *et al.*, 1998). This is about five thousand times

greater than the detection levels estimated for the absorbance example and one hundred times greater than that for standard fluorescence techniques.

### 2.6.2 Relatively temperature insensitive

The fluorescence response is temperature dependent therefore all fluorescence readings should be corrected for temperature (Wilson, Cobb and Kilpatrick, 1986), however fluorescein is less sensitive to temperature than rhodamine B and pontacyl brilliant pink B (Feuerstein and Selleck, 1963). The fluorescence response of photine CU, and rhodamine WT is also more sensitive to temperature than that of fluorescein while lissamine FF, pyranine and amino G acid have fluorescence temperature exponents that are about half that of fluorescein (Wilson, 1968, as quoted by Smart and Laidlaw, 1977).

The fluorescence temperature response of fluorescein has been reported as -0.0036 per degree Celsius using the following formula (Feuerstein and Selleck, 1963):

$$F = F_0 e^{nt}$$

Where F is the measured fluorescence, $F_0$ is the fluorescence at 0°C, t is the measurement temperature (°C) and n is the temperature coefficient ($°C^{-1}$).

### 2.6.3 Relatively low susceptibility to adsorption

Fluorescein is less susceptible to adsorption than a number of other fluorescent tracers (Behrens, 1986 and Smart and Laidlaw, 1977) but there appear to be variables that influence the degree of this adsorption because when Smart and Laidlaw (1977) screened the adsorption of tracers on a range of minerals and organic materials the tracers' performance ranking was similar but the adsorption ratios were quite different.

### 2.6.4 Cost effective

Fluorescein has been found to be more cost effective than the fluorescent tracers rhodamine B and pontacyl brilliant pink B, as long as the time between release and recovery is short: less than 12 hours to 24 hours depending on the water quality (Feuerstein and Selleck, 1963).

## 2.7 Disadvantages of fluorescein as a tracer

The main disadvantages of fluorescein, discussed in detail in the following sections, are that it degrades in light and that its characteristics are pH dependent.

### 2.7.1 Photodegradable

The photochemical decay of fluorescein has been widely noted (Lindqvist, 1960, Feuerstein and Selleck, 1963, Heller *et al.*, 1974, and Wolfbeis *et al.*, 1983). The decay rate is dependent on light intensity, e.g. exposure under cloudy skies gave decay coefficients five times lower than those recorded under clear skies (Feuerstein and Selleck, 1963), and on the chemical composition of the solution as photodegradation does occur in the presence of oxygen but even prolonged irradiation under normal light intensities has no effect on oxygen free solutions (Lindqvist, 1960). Solutions stored in the dark are stable (Diehl and Horchak-Morris, 1987 and Lindqvist, 1960).

Fluorescein's photobleaching rate formula has been reported (Imamura and Koizumi, 1955) as:

$$\frac{-dc}{dt} = \frac{k}{b} I_0 \left(1 - e^{-\alpha c d}\right)$$

Where c is the fluorescein concentration, t is the time, k is the overall rate constant, b is the path length of the light through the solution, $I_0$ is the incident light intensity, and $\alpha$ is the mean molar absorption coefficient. This formula applied up until 70 to 80% bleaching had occurred. Imamura and Koizumi (1955) also investigated the influence of dissolved oxygen on photobleaching by degassing test solutions, adding known quantities of oxygen and then exposing the solutions to light. They showed that the rate constant (k) was not affected by the light intensity, but did increase gradually in the presence of oxygen. Temperature also influenced the rate constant such that $k = 0.3\ e^{-4000/RT}$, where R is the gas constant and T is the temperature.

As fluorescein fluorescence is not directly affected by the presence of oxygen but does photobleach quickly in the presence of oxygen it has been suggested that the fluorescent molecule is not reacting directly with oxygen but that oxygen reacts with one of the photolytic intermediates (Kasche and Lindqvist, 1964).

Apart from oxygen a number of other chemicals have an affect on the photodegradation rate of fluorescein. Ferric ions produce much faster degradation than oxygen (Lindqvist, 1960) while alcohol reduces the rate of photobleaching (Imamura and Koizumi, 1956). Furthermore, the aerobic photobleaching rate is inversely proportional to the alcohol concentration. Additionally if photobleaching takes place under vacuum at a low pH it is irreversible, but in the presence of an alkali, up to 85 % of the photobleaching is reversible when air is introduced (Imamura, 1958).

Despite these reports a number of researchers have commented on fluorescein's good resistance to photolytic decomposition (Melhado *et al.*, 1982, and Sjöback *et al.*, 1995) and some researchers did not detect significant photobleaching in their study and did not think it necessary to degas their fluorescein solutions (Seybold, Gouterman and Callis, 1969).

### 2.7.2 Other reports of instability

A variety of other causes of instability have been reported: Hot aqueous fluorescein solutions were noted to be unstable (Leonhardt *et al.*, 1971), and moderate agitation of solutions inside lightproof flasks produced a drop in fluorescence readings (Feuerstein and Selleck, 1963). Fluorescein has also been suggested to be susceptible to microbial attack (Behrens, 1986) and high sample salinity has been reported to reduce the fluorescence intensity (Feuerstein and Selleck, 1963), however these reports are difficult to assess because the pH of the sample solutions is not reported.

### 2.7.3 pH sensitivity

The pH sensitivity of fluorescein is well recognised (Feuerstein and Selleck, 1963, Klonis and Sawyer, 1996, Martin and Lindqvist, 1975, Smart and Laidlaw, 1977, Lindqvist, 1960, Zanker and Peter, 1958). There are two main affects of this pH sensitivity. The first complication affects the fluorescent or absorbance response and can be eliminated by increasing the sample pH before taking analytical measurements (Feuerstein and Selleck, 1963). The second complication is that tracers that undergo changes in ionic character with pH also experience changes in their sorption characteristics and in the case of fluorescein its sorption coefficient increases as the pH decreases (Behrens, 1986).

### 2.7.4 Other reported disadvantages

Some other disadvantages of fluorescein have been reported as: the need for a UV light source to excite fluorescein at its maximum; its poor water solubility and its susceptibility to quenching by other molecules (Wolfbeis *et al.*, 1983). However it is not clear why a UV light source is listed, as the 490 nm fluorescence excitation wavelength should be within the capabilities of most visible-light sources.

Klonis and Sawyer (1996) mention fluorescein's relatively short lifetime but this refers to the time between irradiation and emission, and should have little impact on its use as a tracer.

### 2.7.5 Summary of disadvantages

There seems to be a number of conflicting observations about the stability of fluorescein. It also appears that it is not just the light intensity but that other variables influence the degradation rate. The obvious conclusion is that if a researcher needs to eliminate this uncertainty, the stability should be measured under field conditions.

## 2.8 Fluorescein analyses

An understanding of spectrophotometry is crucial to understanding the behaviour of fluorescein and the principles of both absorbance and fluorescence will be outlined with reference to fluorescein. The technical terminology adopted here is that recommended by the American Chemical Society (1990) as quoted by Skoog, West and Holler (1992).

Fluorescein can be measured using either the absorptive or the fluorescent character of fluorescein. It is interesting to note that researchers who use fluorescence measurements often quote a single $pK_a$ for fluorescein which implies that it has just two aqueous forms (Smart and Laidlaw, 1977, Wolfbeis *et al.*, 1983, and Grotte *et al.*, 1985), whereas other researchers that use absorbance measurements more readily adopt the multiple species models of fluorescein (Lindqvist, 1960, Zanker and Peter, 1958). Thus there may be important consequences to choosing absorbance rather than fluorescence measurements. The present investigation used absorbance rather than fluorescence readings because spectrophotometers are more widely available than fluorometers therefore absorbance measurements might find broader application.