Development of Marine Phytoplankton...

NRA

National Rivers Authority
Anglian Region

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Anglian Region

Development of Marine Phytoplankton Methodology and Quality
Assurance Procedures

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CONTENTS

1. INTRODUCTION

Professor J.D. Dodge of Royal Holloway College/Algal
Associates was commissioned to write this report in October
1995.

The objectives of the project were to:

- Assess and develop an approach to marine phytoplankton
  sampling and analysis for a variety of needs.

- Identify and compare the available sampling and
  analytical techniques, assess their efficiency and
  comparability. This will enable the selection of
  suitable methods for use in the NRA Anglian Region for
  the production of robust data using standard, tested
  methods.

- Develop suitable quality control procedures for all
  aspects so that the data will be checked giving
  increased confidence in the findings of the surveys.

The methodology was to include discussions with key NRA staff
and review of the available literature. NRA staff and others
contacted are listed in Appendix I.

Phytoplankton is of great ecological significance, comprising
the major organisms involved in primary production. A
knowledge of the distribution of the various phytoplankters in
both space and time is essential for an understanding of a
particular ecosystem. The seasonal succession of species has
an effect on the components higher up the food chain and is
consequently important for economic reasons.

In addition to their significance in the food chain,
phytoplankton contain species recognised as potent toxin
producers. These microalgal toxins have been classified as
paralytic shellfish poisoning (PSP), diarrhetic shellfish
poisoning (DSP) and amnesic shellfish poisoning (ASP). The
increase in reported cases of algal toxins has led to
speculation that industrial pollutants, agricultural and waste
water run-offs may be to blame (Anderson, 1984; Hallegraeff,
1993). Toxicity of phytoplankton can be transferred to
suspension feeders rendering shellfish growing in red tide
areas suspect of being dangerous for human consumption. The
toxicity associated with red tides may also be felt in the
effects of sea spray on man and in fish mortality rates.

Besides knowing which species are present at different
locations and seasons, a knowledge of the total abundance of
phytoplankton is necessary for a better understanding of the
ecosystem and its dynamics. This can be measured and
expressed as cell numbers in samples of sea water. Such a

method of measuring phytoplankton populations is not without its problems as cell size can be variable not only interspecies but even intraspecies. The significance of cell size variability is that species which are unimportant numerically may be important in terms of biomass and such a numerical census may overestimate small cells and underestimate the contribution of large cells (Paasche 1960). Biomass can be measured from proximate analyses, typically chlorophyll or indirectly from the cell-volume characteristics of an enumerated population. For most purposes speed of analysis is essential for obtaining a good picture of a current situation.

A degree of standardisation of methods is necessary to obtain the best use of results. Regular quality control is required to ensure that results of acceptable quality are being obtained in a subject area which is renowned for the difficulties of analysis.

For EC UWWTD and the Nitrate Directive, in relation to eutrophication, it has been recommended "that a primarily observational approach is adopted, with the aim of establishing the occurrence of exceptional nuisance algal blooms, the duration of blooms, excessive macro-algal growths, or other evidence of undesirable disturbances" (NRA Programme for the Monitoring of Water Quality, Nov 1994, Annex 6 UWWTD). This should be undertaken at existing monitoring points, notably those for bathing waters and shellfish waters.

All EC bathing waters are monitored weekly, May to September inclusive, as part of the National Marine Algal Bloom Monitoring Programme. On occasions where algal blooms are seen to be present, samples should be taken to confirm the bloom. For sites where regular 'significant blooms' occur monthly summer sampling is recommended. Consideration should be given to remote sensing surveys, from aircraft, at such sites to determine spatial distribution of blooms and provide evidence of impact." (NRA Guidance Note on Information Gathering for Future Designation Reviews UWWTD August 1994. In: NRA Programme for the Monitoring of Water Quality, Nov 1994, Annex 6).

Plankton, as defined by Hensen in 1887, can be summarized as those organisms, both plant and animal, that 'drift' passively in water. Many of these organisms are able to swim, but because of their small size progression through the water is slow and their distribution is often patchy and determined by the movement of currents and tides.

Phytoplankton is predominately unicellular algae which are either solitary or colonial. The main components of marine phytoplankton are diatoms (Bacillariophyta), dinoflagellates (Dinophyta or Pyrrophyta), coccolithophorids and chrysomonads (Haptophyta) and some other flagellates. Phytoplanktonic organisms are autotrophic containing photosynthetic pigments such as chlorophylls and carotenoids. Some phytoplankton

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organisms, mainly dinoflagellates, can be heterotrophic, building up organic particulate matter from dissolved organic substances (osmotrophy) or even particulate organic matter (phagotrophy). Some taxa, such as *Protoperidinium* are classified as both phytoplankton and zooplankton (protozoa).

Autotrophic phytoplankton is most abundant in the euphotic zone, defined as the zone reaching from the sea surface to a depth where the production of organic matter by photosynthesis in an individual phytoplankton cell balances destruction by respiration.

Phytoplankton is not evenly distributed in the sea but is believed to occur in three-dimensional patches of various sizes.

Cell size and volume cover a wide range from one to 200µm. Picoplankton is the term used for the smallest cells of less than 2µm, nanoplankton (or nannoplankton) for those cells between 2 and 20µm, and microplankton (or net-plankton) for those between 20 and 200µm. Plankton nets can be used to concentrate the larger phytoplankton species, but picoplankton and nanoplankton normally passes through phytoplankton nets. Sedimentation or filtration methods are used to collect these organisms. In general the use of plankton nets underestimates the total standing stock of phytoplankton.

2.   REQUIREMENTS

2.1   Introduction

Monitoring of marine phytoplankton by the NRA is undertaken to comply with the Water Resources Act (1991), and EC Directives, to assess eutrophication,and to identify toxic and nuisance species.

2.2   UK Legislation

The NRA was brought into existence by the enactment of the Water Act 1989, later superseded by the Water Resources Act 1991.   The Water Resources Act requires the NRA to "monitor the state of pollution in *controlled waters*". Controlled waters comprise surface freshwaters, groundwaters, estuaries and coastal waters to the three-mile limit, in England and Wales. The Water Resources Act (1991) states that water samples should be taken throughout the period 1 May to 30 September, with additional samples taken if the water is or is likely to deteriorate.

The Environment Act 1995 establishes the Environment Agency, due to come into existence in April 1996, which will take over the functions of the NRA, Her Majesty's Inspectorate of

Pollution and the waste regulation responsibilities of local authorities, to provide an integrated pollution control regulation in England and Wales.

There is also the North Sea Action Plan for which work has been incorporated into the National Marine Monitoring Programme (NMMP) for chlorophyll *a* analysis and phytoplankton sampling.

2.3   EC Legislation

The relevant EC Directives are:

- Urban Waste Water Treatment Directive [91/271/EEC],

- Bathing Water Directive [76/160/EEC]

- Nitrate Pollution Directive [91/676/EEC].

- Shellfish Waters Directive [79/923/EEC]

The Urban Waste Water Treatment Directive was introduced to protect waters from the adverse effects of waste water from sewage treatment works, and certain sectors of industry. It specifies secondary treatment for all discharges serving population equivalents greater than 2,000 to inland waters and estuaries, and 10,000 to coastal waters. It lays down minimum standards for Phosphorus and Nitrogen sewage discharges to "*sensitive areas*", that is, areas such as estuaries and coastal waters "which are found to be eutrophic, or which in the near future may become eutrophicated if no protective action is taken".   The NRA's role is to gather data in support of proposed "*sensitive area*" designation. A major limitation of the directive is the definition of "eutrophication". The directive defines it as " the enrichment of water by nutrients, especially compounds of nitrogen and/or phosphorus, causing accelerated growth of algae and higher forms of plant life to produce an undesirable disturbance to the balance of the organisms present in the water and to the quality of the water concerned".   Anglian Region has no designated coastal Nitrogen limited Sensitive Areas.

There is a stated requirement for good international laboratory practices to be employed, aimed at minimizing the degradation of samples between collection and analysis. The Directive does not detail the methods to be employed for sampling or analysing phytoplankton, in the way in which chemical analyses methodologies, for oxygen or suspended solids, are outlined.

The Bathing Waters Directive sets a range of standards which must be achieved in designated bathing waters. During the season May to September there is a requirement for fortnightly sampling of water to measure total and faecal coliforms per

100 ml, faecal Streptococci, Salmonella, Enterovirus, pH,
colour, mineral oils, surface active substances reacting with
methylene blue, phenols, transparency, dissolved oxygen, tarry
residues, plastic bottles etc, ammonia, Kjeldahl nitrogen,
pesticides, heavy metals, cyanides and nitrates.

Under the terms of the Nitrate Pollution Directive surface
water is to be sampled and the eutrophic state of fresh
surface waters, esturial and coastal waters reviewed every
four years.

The Shellfish Waters Directive is concerned with the quality
of shellfish. Saxitoxin, produced by dinoflagellates, is
required to be measured.

In 1992 there was a Council Resolution on the future Community
Policy concerning the European coastal zone. This recognised
the fragile nature of the European coastal zone and said it
was "essential that its biological diversity, ecological
quality and capacity to sustain life, health, social
activities and social well-being are safeguarded".

2.4   Phytoplankton Monitoring

Phytoplankton is therefore monitored for different reasons and
the sampling method depends on the objectives of the study.
Whatever the aims of the investigation the samples collected
should be representative, as far as is possible, of the whole
water body being studied. This implies a careful consideration
of water flow and tidal state, particularly in estuarine and
near-shore areas. In open sea areas, provision should be made
to allow for an adequate number of samples to be taken to
allow for the possibility of patchiness in the occurrence of
phytoplankton. Wherever possible a larger initial sample
should be taken than needed and one or more subsamples taken
from this after thorough mixing.

Chlorophyll analysis of sea water, either from laboratory
analysis of filtered samples, continuous flow fluorometry, or
aerial surveillance, can give valuable information,
particularly when used in conjunction with species
identification and other chemical analyses.

The quality of the results depends on the method of collection
of samples, and their subsequent subsampling, their analysis
and the skill of the operators at each stage. To ensure that
these processes are carried out in the best cost-effective
manner requires quality evaluation at each stage.

3.   SAMPLING

3.1   Introduction

The sampling method employed is determined by the objectives
of the survey and the methodology of analysis. These
objectives may be varied and include the production of a
species list, an indication of abundance of certain species or
for a calculation of chlorophyll present. In order to
determine cell numbers or biomass it is necessary to collect
the abundant forms of all sizes as well as the rarer large
cells, which because of their size may be an important
constituent of the biomass.

The nature of the distribution of phytoplankton, that is its
patchiness, vertical differences and diurnal changes, poses
problems for statistical analysis. Regular sampling and
analysis throughout the year, but at more frequent intervals
during the spring and autumn blooms is necessary for an
understanding of the ecosystem. The sampling programme
employed must be practical in terms of manpower available to
analyse the samples and flexible enough to be able to adapt to
changing conditions, such as the development of 'red tides'.

The concepts of accuracy and precision are distinct and not
necessarily synonymous (Venrick, 1978). Net tows, for example,
may give precise estimates of abundance but have poor accuracy
because they underestimate the true abundance of phytoplankton
as the smaller cells are only partially retained by the mesh
and others may be destroyed during the fixation process.

Long-term studies are essential to illustrate trends in
eutrophication, although the numbers of samples taken may be
reduced with experience or where good correlations have been
established. One possible example might be an aerial
surveillance system superseding water sampling for pigment
analysis, although financial considerations might render this
impractical. Similarly, if a good understanding of the species
composition at different times of the year exists, automatic
counting devices could be used to reduce the number of
plankton samples counted manually.

Experience gained from sampling sites over several seasons is
important for sampling programme design, especially in
estuarine situations, where a marked change in chlorophyll
concentrations near the halocline or thermocline can be
expected (Yentsch, 1965) as well at different stages of the
tide (Welch and Isaac 1967).

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3.1.1.   Treatment of Samples

It is normally desirable to examine fresh material, both for biological and chemical analysis, in addition to preserving material. Some species can be identified reliably only from live specimens. Ideally live samples should be examined on the day of collection. If this is impractical, they should be stored in the dark in a refrigerator or cool place (normally at sea temperature), then looked at within 24 hours. Fixation of samples not only distorts cell shape but reduces the size of cells. The very small flagellates frequently burst when a fixative is added and are lost for identification and analysis, resulting in an underestimate of biomass.

Fresh sea water samples for chlorophyll analysis should be kept cool and dark and sent to the laboratory for examination with the minimum delay. In estuarine waters chlorophyll concentrations vary with the stage of the tide so this should be borne in mind when samples are taken for comparative purposes.   There are also diurnal variations to be considered (Yentsch and Scagel 1958).

3.1.2   The Importance of Salinity

Salinity has a considerable influence on what species are present and their distribution.   Consequently, in estuaries and inshore areas a decision needs to be taken whether to sample from fixed sites or, alternatively, in relation to salinity isohalines.

3.1.3   Sampling Strategies

A purely descriptive programme does not have the stringent requirements of a statistical one.   Any quantitative programme must have:

   (i) a rigorous statement of the objectives and

   (ii) a definition of the target population (Cochran, 1963).

In reality, the conditions required by classical statistical methods are rarely, if ever, realised in the planktonic environment (Ibanez, 1978). The three dimensional nature of marine phytoplankton has been referred to above and in addition the difficulties of executing a specific sampling strategy on an invisible, motile population are huge. It is preferable to plan for long-term studies as they can show sustained trends due to climatic change and the effects of eutrophication or other cultural effects (Margalef, 1978). Eutrophication is defined in the UWWT Directive (91/271/EEC) as "the enrichment of water by nutrients, especially compounds of nitrogen and /or phosphorus, causing accelerated growth of algae and higher forms of plant life to produce an undesirable

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disturbance to the balance of organisms present in the water
and to the quality of the water concerned".

3.1.4   Quality Assurance

  •   The sampling strategy should meet the needs of the
investigation.   These may change over time to allow for
altered circumstances.   A clear understanding of the
objectives is essential for the best sampling programme to be
in operation.

  •   For long term studies sampling from as near as possible
the same location is required.   The marking of locations with
buoys has been thought to influence the composition of the
phytoplankton around it (Léger 1971 a, 1971 b).

  •   Sampling precision and accuracy should be evaluated
regularly.

  •   All sampling equipment should be kept clean.

3.2   Sampling Methods currently used by the NRA (Anglian Region)

As part of the National Coastal Surveillance Programme,
initiated by the NRA's National Centre for Marine Surveillance
at Twerton, Bath, the Region has a purpose-designed coastal
survey vessel the *Sea Vigil*.   Surface water (0-27 m depending
on location) samples are collected from 34 sites with data
collected at approximately monthly intervals. Samples are
immediately filtered and analysed on-board for nitrate,
ammonia, phosphate, silicate and nitrite, using an auto-
analyser. The methods used contain built-in Analytical Quality
Control procedures and have passed special marine AQC
exercises, particularly the stringent ones prepared for ICES
(International Council for the Exploration of the Seas).
Measurements of temperature, salinity and dissolved oxygen are
also made. Chlorophyll *a* samples are filtered on-board,
preserved and sent to the Ipswich biology laboratory for
analysis. There is a lack of historical data to make
comparisons with but as the programme continues trends will
become discernible.

The NRA employs a variety of sampling methods throughout its
regions.   Water samples fixed in Lugol's Iodine is the most
used sampling method in all three areas.   Unfixed water
samples are less commonly taken.   Net samples are taken by the
NRA, Ipswich (Eastern Area). Intertidal substrate sampling is
undertaken by Eastern Area.

Water samples for chlorophyll analysis are taken, but this is

mostly carried out on-board with an autoanalyser.

The integrated monitoring and logging system developed by the NRA's National Centre in Bath uses a QUBIT TRAC V Navigation package on its marine survey vessels. The Centre sets a common specification for all four coastal survey vessels, ensuring that all data can be integrated into a national classification programme. The parameters measured include:

- Conductivity, Temperature and Depth
- Salinity
- Dissolved Oxygen
- pH
- Transmission
- Fluorescence
- Plankton Sampler
- Saline Nutrients

3.3   SAMPLING TECHNIQUES

3.3.1   Water Samplers

Water samplers should collect a known quantity of water from a specified depth and keep the sample free from contaminants.

3.3.2   Surface Samples

For surface water a bottle or bucket is lowered into the water and allowed to fill before recovery. To avoid contamination from the research vessel, or from disturbed sediment when collecting from the shore, the bottle may need to be weighted, have a surface float attached and also a line and be tossed free of the collector, allowed to fill and then hauled back.

Most commercial bottles are now constructed from or coated with an inert material, such as teflon or PVC to eliminate chemical contamination, including rust and paint flakes. However, if physiological work is to be carried out it may be necessary to avoid rubber and plastic, which may be toxic to some phytoplankton species (Throndsen, 1970). Water bottles are available in sizes from 1-1000 litres, but most laboratories have found 1-3 litre bottles are adequate.

Samples for chlorophyll analysis should be filtered immediately but if this is not possible they should be stored in sealed containers in the dark, kept cool (in a cool box or refrigerator), and transported to the laboratory as soon as possible.

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3.3.3 Tube Samples

For sampling the top 5 metres or less an integrated sample can
be obtained with a length of hose or flexible tubing, which is
attached to a line and weighted at the end that is lowered
into the water to the desired depth. The surface end is then
sealed with a clip or plug and the lower end raised to the
surface with the line. A 2.5 cm internal diameter tube of 5 m
length gives a sample of over 2 litres. This quantity would
be sufficient for chlorophyll analysis and microscopical work.
(For further details see Lund *et al* 1958; NRA Standard
Methodologies: Freshwater Phytoplankton, 1993.)

3.3.4 Profile and Fixed Depth Samplers

The NRA takes water samples which are then fixed with Lugol's.
They use 25 ml, 150 ml or 1 litre samples.

Samples from predetermined depths are taken with either a
reversing-bottle of the Nansen type (Helland-Hansen and
Nansen, 1925), a non-reversing bottle, Van Dorn (Van Dorn,
1957) type or a sampler which opens only at the required
depth. Simultaneous sampling at several depths permits savings
in time and effort over a single bottle lowered repeatedly to
different depths. The design most frequently used is a
plastic or metal cylinder which is lowered to the required
depth with both ends open. Closure is usually triggered
mechanically by a messenger. In reversing bottles, such as the
Nansen bottle the messenger strikes and releases the upper
clamp, allowing the bottle to swing 180° about the lower
clamp. This reversal closes valves in the top and bottom of
the bottle. In non-reversing bottles, such as the Van Dorn the
top and bottom caps are held open by a clamp against the
tension of a spring or rubber connecting them through the
bottle. The action of the messenger releases the clamp, and
the caps are pulled into position, closing off the top and
bottom of the bottle (Venrick, 1978).

There are other water bottles which are designed to reach the
desired depth in the closed position and are then opened.
These have been produced primarily to avoid contamination from
the surface layer and descriptions of their use are in Aabye
Jensen and Steemann Nielsen, 1953, Niskin, 1962 and Throndsen,
1970b.

Horizontal samplers, designed to sample a narrow stratum are
useful in very shallow water, or for epibenthic samples
(Joeris, 1964; Ottman, 1965)

3.3.5 Quality Assurance

   ·   All sampling bottles, hoses and nets should be rinsed
after use to avoid contaminating samples.

· Samples must be clearly labelled with waterproof marker. Where possible physical data, such as temperature and salinity, should also be marked on the bottle. Where wet conditions preclude this a paper label written in pencil should be inserted in the sample bottle.

· A record book stating the location and time of sample should be kept.

· Live samples should be kept cool, in a cold box or portable refrigerator, and transported with the minimum delay to the laboratory. They should be kept in the dark.

· For comparative purposes there must be consistency in the sampling method. Personnel engaged in these activities should attend appropriate training sessions if possible.

· Sufficient bottles for the sampling programme, plus a few extras to account for any losses or unexpected phenomena, such as red tides, should be taken.

3.4   Nets

Sampling with phytoplankton nets provides useful material for qualitative purposes but because of their selective and unpredictable filtering properties they should not be used for quantitative purposes (Braarud 1958). The main advantage of nets is the large quantity of water which can be filtered for concentrating cells. The chief disadvantage is the distortion to species composition resulting from loss of very small cells. The filtering qualities of the net can be affected by the species composition of the plankton. Chain-forming species, or those with spines or setae (e.g. *Chaetoceros* spp.) can themselves form a network inside the gauze. A vertical or horizontal net haul is a quick and effective method for collecting rarer, larger species. Where there is a large quantity of suspended debris the net quickly becomes clogged.

Nets constructed for vertical hauls are weighted on the line before the mouth of the net. Modern nets are made of synthetic filaments such as polyamide or polyester. Fine mesh gauzes have low porosity and so a compromise has to be reached between filtration and species concentration and size of species collected.

For general purposes it is recommended that a net with meshes of 53µm be used. This is the standard mesh used in most phytoplankton nets, and has relatively good porosity (See Table 1 below). Nybolt is a monofilament nylon fabric, which although having limited resistance to acid shows no evidence of toxicity, is stable to ultraviolet radiation (there is some loss in tensile strength after long time exposure), and is resistant to decay and bacteria.

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Table 1
Porosities of some Nybolt monofilament nylon gauzes

Mesh size µm	Thread diameter (warp/weft) µm	Porosity (free sifting surface) %
100	43	49
80	43	42
60	37	39
55	37	34
53	30	35
50	39	27
48	35	31
40	30	30
30	30	23
25	39	14
20	30	12
15	30	10
11	30	4
5	35	1

Source: Plastok Associates Ltd., Birkenhead, UK Supplier of Schweizerische, Seidengazefabrik, AG Zurich, Switzerland, and manufacturer of bespoke plankton nets.

A semi-quantitative study can be achieved if there is consistency in sampling. For vertical hauls the net should be lowered to the same depth and raised at the same slow speed each time. If the weight is attached behind the tail of the net the net does not filter on the way down but only when being hauled up. Horizontal tows should be made at the same speed for the same time. A plankton sample from a particular depth is obtained by towing the net horizontally while the weight holds the net at the selected depth. Care should be exercised to limit speed when towing a net. For nets of about 50µm mesh this speed should not exceed 1 m. s^{-1} or 2 knots; nets with meshes below 20µm should be towed at speeds below 0.3 m. s^{-1} or 0.5 knots. A flowmeter can be fitted in the mouth of the net to enable a calculation to be made of the amount of water passed through the net. Nets which are equipped with a non-filtering cone in the front or long nets may be towed at slightly higher speeds than the standard net of the same porosity; the low net-mouth/gauze-area ratio in these nets results in reduced pressure on the gauze (Tangen,1978).

Although the collecting bucket at the end of the plankton net receives most of the plankton during towing some cells remain on the gauze and should be washed into the bucket at the end of each haul with seawater. Filtered seawater should be used to wash the gauze when the net has been used for a deep-water tow using an opening-closing net to avoid contamination from surface organisms.

Net hauls should not be too dense, and they should be kept in a large amount of water (250 - 1000 ml) if they are to be

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examined live. They should be kept cool (2 - 5° C) to minimise
bacterial growth. Dense samples from nets, as well as those
from blooms, decay rapidly and should either be diluted with
fresh seawater to extend viability or fixed immediately after
collection.

3.4.1 Quality Assurance

• Nets should be washed as soon as possible after use, and
should not be allowed to dry before washing. They should be
washed in tap water to remove salts. After washing the net
should be air dried and stored in a dark, cool place. It
should be inspected for damage before further use.

• Nylon nets can be cleaned occasionally by soaking in
warm soap or detergent solution or an alkaline solution up to
15% strength. Polyester nets should be washed in dilute acids
instead of alkaline solution but in general soap will be
adequate for cleaning although benzene or acetone may be
necessary to remove extensive clogging by organic compounds
such as oil. After soaking rinse thoroughly in clean water
before air drying and storing in a dark, cool place.

3.5   Pumps

Samples from selected depths may be obtained by pumps. Pumped
water volumes can be accurately measured with a water meter
for quantitative studies, however frictional resistance may
inhibit the movement of some organisms. Samples can be
concentrated by filtering through a plankton net on board
ship. Pump systems are normally more expensive than nets and
bottles but can prove cost effective when sampling for a
variety of zoo- and phytoplankton taxa together with chemical
analysis.

3.5.1 Quality Assurance

• Pumping systems must be tested for toxicity,
particularly when the samples are used for live organism
studies.

• The growth of organisms in the tubing can be reduced by
washing with fresh water.

3.6 Bathing beach samples

Samples should be taken if there is any sign of an algal
bloom, scum or if the water or beach surface is discoloured.
This should be done with the minimum of disturbance to the

site by gently lowering bottles to sample the surface.
Normally two samples are taken of 250 ml each, of which one is
kept live and the other fixed with Lugol's Iodine. These are
kept cool, packed in light-proof containers and sent or taken
to the laboratory for analysis. Following analysis a Toxic
Algal Bloom Report should be completed and sent off according
to the Marine Algal Monitoring Programme, V. 2, 1994 (Appendix
V).

Samples of the substrate are taken whenever bloom deposits are
found on a beach. The sample is taken with a flexible scraper
from at least 5 cm^2. A measured sample can be taken in this way
for quantitative studies. The sample should be kept cool and
dark and examined microscopically as soon as possible.

Accumulations of floating scum should be sampled where they
are within safe sampling distance of the shore. Enough
material should be taken to fill a sample tube and a duplicate
should be fixed with Lugol's Iodine if it is not possible to
examine the sample within an hour tow of collection.

3.7   Subsampling

The original sample should be thoroughly mixed before
subsamples are taken. The size of the subsample depends on
the material to be examined and the aim of the investigation.
Hasle, (In: Sournia 1978) suggests shaking a fixed sample 100
to 200 times by hand to loosen organisms attached to the
bottom of a storage bottle before subsampling. For fresh
samples and those with little detritus it should be sufficient
to shake or invert the bottle 20 to 30 times to disperse
particulate matter evenly. When subsamples are taken by
pipette the tip should be sited as near the centre of the
sample bottle as possible.

3.8   Recommendations for sampling

The NRA has a requirement to monitor marine plankton as a
means of monitoring pollution and environmental quality. It is
recommended that where possible a phytoplankton sampling
programme is developed in conjunction with zooplankton
sampling and chemical analysis programme. A more
comprehensive understanding of the environment year on year is
possible if, for instance, analysis of chlorophyll and other
chemicals in the water can be related to identified species
and their abundance.

Live samples are often necessary for accurate identification,
particularly for non-thecate organisms. Where fixatives are to
be used these should be added as soon as possible after
collection.

Samples for chlorophyll analysis must be treated carefully to

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avoid breakdown of the pigments.

4. FIXATION METHODS

4.1 Introduction

The handling of samples after collection is a critical stage
in most phytoplankton work and it is important to minimise
quantitative and qualitative changes in the composition. This
is achieved by fixation of the sample(s) or by keeping them at
a low activity rate. The requirement for live samples for
identification of certain organisms, such as naked
flagellates, should not be ignored.

There is no single fixation method which is suitable for the
preservation of all types of phytoplankton (naked algae,
armoured dinoflagellates and the calcareous organisms). Pico-
and nano-plankton are now recognised as being an important
constituent of biomass (Hallegraeff, 1981; Booth *et al*, 1982;
Furuya and Marumo, 1983; Murphy and Haugen, 1985; Davis *et al*,
1985). Most fixation methods normally used for phytoplankton
distort, shrink or destroy these delicate organisms.
Measurements made of different nanoplankton species live and
after fixation have been made by Booth (1987). He showed that
cells preserved with Lugol's were the closest in size and
shape to live cells, whereas those preserved with formalin
were the most distorted and shrunk, with many cells bursting.
There was significant shrinkage (between 2 and 36%, average
15.7%) in cell length in cells fixed with glutaraldehyde.

The effect of fixation and preservation should be checked by
comparing counts of preserved subsamples with those of live
subsamples, or with the results of autoradiography (Magure and
Neill, 1972; Watt, 1971). Comparisons of biomass estimates
from cell counts with photosynthetic rates in other subsamples
are also useful to test results.

4.2 Glutaralderhyde

When examining live samples a gentle "fixing" agent, such as
glutaralderhyde may be used if swimming disturbs the
examination/counting (Throndsen 1996). This fixative should
also be used ~~should~~ later examination by a taxonomic expert,
possibly using electron microscopy, be contemplated (e.g. as
in the case of a particularly unusual occurrence), or for
cryo-preservation (Booth, 1987).

4.3 Lugol's Iodine

Fixation should take place immediately after sampling. The recommended fixative for routine sampling is Lugol's Iodine (100 g potassium iodide dissolved in 1 litre of distilled water and then 50 g crystalline iodine dissolved and 100 ml glacial acetic acid added). Use the minimum required to give water samples a weak brown/straw colour, between 0.4 and 0.8 ml per 200 ml sample (Willén 1976), and shake well. Containers should be clear glass bottles, as coloured glass would make it difficult to gauge the correct amount of Lugol's. Plastic bottles take up iodine from the solution and so when stored for a long period samples should be checked for iodine concentration. If this is low add more Lugol's. When there is abundant detritus or phytoplankton the Lugol's is absorbed quickly. Samples should be inspected after 24 hours to see if more Lugol's is required. Note: Light increases the loss of molecular iodine from samples.

Lugol's has the advantage over formaldehyde of preserving the flagella on many flagellates. The cells stain brownish yellow aiding counting, when there is not too much particulate matter. The main disadvantage is that coccoliths and also silica will dissolve with long storage.

Note that this method is suitable only for water samples; net hauls being preserved better in formaldehyde.

4.4 Formaldehyde

The agent is a 20% aqueous solution of formaldehyde neutralised with hexamethylenetetramine (100 g hexamethylenetetramine to 1 litre of the 20% solution). For water samples 2 ml of this solution is added to each 100 ml sample and the sample shaken gently immediately to facilitate fixation. For net hauls sufficient solution is added to the sample to make up about one quarter of the volume, in order to give a final concentration of 4-5% formaldehyde. Once preserved the samples will keep for years if stored in a dark, cool, place.

A disadvantage of using formaldehyde as a fixative/preserving agent is that it distorts the cell shape of naked species and causes flagella to be thrown off in many flagellates. Bleaching of cell contents may occur rendering it difficult to distinguish between pigmented and non-pigmented cells.

4.5. Cryo-preservation

This method has been described as being useful for storing samples for later use of epifluorescence microscopy (Booth, 1987). It is not recommended for routine analysis.

For water samples containing <1 mg chlorophyll a m^{-3} 50 ml
aliquots of sample are fixed (glutaraldehyde, 2.5% final
concentration) in a filter funnel over a 0.4 μm Nuclepore
filter stained with Irgalan Black, for 20 minutes. After
filtering the filter is mounted on a drop of immersion oil
(Cargille Type B, non-fluorescent) and another drop added on
top of the filter and a coverslip put on top. The filters are
stored in the dark at 4°C for a few days while the water is
replaced with oil and then stored in a freezer (-20°C).
Samples stored in this way will keep for two years or more
without significant loss of cells.

5. ANALYSIS: METHODS AND EQUIPMENT

5.1 Introduction

The chosen method of analysis is determined by the aim of the
study. Samples collected for quantitative examination can be
grouped into those used for estimates of cell numbers or
biomass and those which will be used for assessments of all
cells and species.

Cells may be counted with a Coulter counter or flow cytometer,
although these methods are best suited to detritus-free
conditions.   They have the disadvantage of not providing
species identification, or evaluation of appearance such as
colony formation or size.   Use of a standard light microscope
or inverted microscope such as the Zeiss Axiovert 10 is
preferable. Where the population is composed of many species
of different size and abundance a combination of methods may
be employed, such as settling chambers examined with an
inverted microscope and counting slide on a standard light
microscope.

5.2 Inverted Microscope (Utermöhl method)

The Utermöhl technique (Utermöhl, 1931, 1958), or as it is
referred to sometimes less correctly, the sedimentation
method, is the preferred method for identification and
enumeration of phytoplankton (for species which are
identifiable in preserved condition), particularly for cell
concentrations in the $10 - 10^6.16$ l^{-1} range. For higher
concentrations samples can either be diluted or an alternative
method employed, such as Palmer-Maloney counting chambers or
haemacytometers particularly when cells are very small.

The method uses an inverted microscope, such as the Zeiss
Axiovert 10 or Wild M40, which has the light source and
condenser above the chamber and objectives which view the

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settled specimens from underneath. The microscope is fitted
with a mechanical stage, with vernier scales. The stage is
designed to take Hydro-Bios counting chambers of sizes from 2
to 10 ml. Ideally there should be three x10 eyepieces; one
with a Whipple graticule and one with a cross-hair graticule.
The various squares of the Whipple graticule are calibrated
using a micrometer slide and the results tabulated and kept
with the microscope. The cross-hair graticule is calibrated by
measuring the distance between the two side hairs so that when
multiplied by the diameter of the sedimentation tube the area
of one traverse can be calculated. Magnification is required
at x6.3 or 10, x20 phase objectives, x40 phase and x100 phase
water or oil immersion.

5.2.1 Counting Chambers

A number of 2 ml, 5 ml and 10 ml chambers should be available;   x
sufficient for the scope of the work proposed and taking into
consideration the time taken to settle samples, which can be
as long as 24 hours. The Hydo-Bios type are recommended. Each
should be indelibly marked with a reference number. Some
workers have devised chambers with a smaller floor area than
the standard in order to concentrate the sample (Throndsen,
1970a). These are useful for studies where the whole floor
area is examined and when small organisms are to be
enumerated. In coastal and estuarine areas with a high
quantity of suspended particles a wider surface area is
preferred.

Combination plates, with a detachable sedimentation cylinder
fitted into a bottom plate which holds the settled sample,
have been developed to allow a relatively large sample to
settle, without rendering the use of a condenser impossible.
(This had been the disadvantage of Utermöhl's original
design). The top cylinder holds 10, 50, or 100 ml and after
sedimentation it is removed. The bottom-plate chamber consists
of a rectangular perspex plate, a ring and a circular base
plate of coverslip thickness. The plate fits into the
mechanical stage of an inverted microscope. Combination plates
have the disadvantage of being expensive, unreliable and
difficult to use, although they can save time by settling a
larger quantity of water. In coastal and estuarine waters with
large quantities of detritus this advantage is of no value.
Their use is not recommended for routine work.

Hydro-Bios chambers consist of a threaded metal base plate
with screw-in 'plexiglass' tubes, which hold a glass cover-
slip, through which the sample is viewed, in place. If leakage
occurs through the cover-slip it can be sealed with a smear of
grease, such as Apiezon AP101, applied carefully to the base

plate. This should then have the chamber screwed in and excess grease removed before dismantling and fitting the cover-slip.

Judgement should be used to determine the ideal sedimentation volume. This should be determined by phytoplankton density, amount of detritus and purpose of the study. Experience will help with the determination of the volume, colour of the sample previous records or chlorophyll a data. For an initial study settle samples in a range of chamber sizes to determine ease of counting. Whatever volume is selected clear records must be kept for each sample showing:

    Sample location
    Sample site
    Date of sampling
    Chamber number
    Sedimentation volume
    Sedimentation date
    Sedimentation time

A sample with a sparse population and little detritus is best examined in a chamber with a bottom area relatively small to the volume. Samples with a high density of detritus, frequently found in estuarine and turbulent inshore waters, are more easily examined in a chamber with a large bottom area relative to volume. It may also be necessary to dilute such samples.

Tangen (1976) found that an exactly horizontal table, one fitted with a spirit level, assured a more homogeneous distribution of the sediment on the chamber floor, and reduced leakage.

The sedimentation time is determined by the fixative used, the volume of the sample in the chamber and the size of the organisms in the sample. The time taken to allow samples to settle is normally at least one hour per millilitre (Lund et al, 1958) for small chambers but 3 hours is often adequate for a 10 ml chamber. Providing sufficient chambers are available, it is preferable to standardise settling on a longer time, such as overnight (approximately 12 hours) for all samples.

Incomplete sedimentation can result when cells become attached to the chamber walls, convection currents occur in tall chambers, or because cells do not gain sufficient weight when preserved to settle. Lugol's fixed samples settle quicker than formalin fixed samples since the iodine increases the density of the plankton cells; this method does however destroy coccolithophorids. Hasle (1978) has reported that some species, such as Chaetoceros sp. are not distributed randomly on the bottom of the chamber, but tend to accumulate around the periphery. Care must be taken to ensure that when counting only a part of the bottom area this is taken into account.

5.2.2 Quality Assurance

- The microscope should be kept clean and covered when not in use.

- Lenses should be cleaned with lens tissue.

- The microscope should be serviced annually.

- Samples should be at room temperature before filling the chamber to prevent air bubbles forming on the chamber walls.

- During the settling period the temperature should be fairly constant to prevent convection currents interfering with sedimentation. Similarly exposure to sunrays light should be avoided.

- Chambers should be cleaned immediately after use with a jet of water from a wash bottle. They should be wiped dry with a medical wipe and stored inverted or covered.

- The glass cover-slips of chambers should be replaced regularly.

- Clear records must be kept with the chambers to avoid confusion.

5.3 Standard Light Microscope

5.3.1 Counting Slides

Living cells are best identified and counted with a light microscope and examined on a counting slide such as a Sedgwick-Rafter or Palmer-Maloney Counting Slide or, where the plankton consists of very small sized cells (<20µm), with a standard haemacytometer. When examining live samples a gentle "fixing" agent, such as glutaralderhyde or the short term fixative Uranyl acetate, may be used if swimming disturbs the examination/counting (Throndsen 1995). This method is not recommended for routine samples but only for *ad hoc* qualitative examination.

Any cells overstained by iodine, following the addition of Lugol's, may be cleared by adding a drop of saturated solution of sodium thiosulphate.

Throndsen (1996) recommends Palmer-Maloney counting slides for routine surveys. He recommends a standard microscope fitted with x10 x20 and x40 objectives and preferably with phase contrast optics. A x100 oil immersion objective will be necessary for identification of the smaller organisms. A Whipple graticule should be fitted in the eyepiece, calibrated with a ruled microscope slide. As with the chambers for the inverted microscope a settling time is necessary for counting

slides. A Palmer-Maloney chamber holds 0.1 ml, whereas the
Sedgwick-Rafter slide holds 1 ml. (The microscope should have
a mechanical stage.) Haemacytometers come with grids marked on
them and so ocular grids are unnecessary.)

Before counting, examine the chamber under low power to detect
obviously unsatisfactory distributions of cells. Counts can be
made of the whole chamber or in a given number of randomly
selected Whipple fields or grids.

5.3.2 Quality Assurance

•   Counting slides should be scrupulously clean. After the
removal of samples with distilled water they can be cleaned
with ethanol, 'denatured' alcohol or acetone and dried with a
medical wipe.

5.4 Identification and counting of plankton in water samples with a high detritus content

These should be diluted and settled in 5 ml tubes in order
that cells are visible. The degree of dilution depends on the
amount of sediment in the sample but should be recorded so
that results can be related to the original sample size.

6   ANALYSIS: ENUMERATION TECHNIQUES

6.1 Introduction

Success of counting depends to a large extent on the chosen
sampling programme. Normally counts are made from subsamples
of the initial field sample. Phytoplankton cell
concentrations can vary enormously and an individual species
may be rare but of significance rendering its detection
important. For this reason it may be necessary to concentrate
the original sample.

In nature the actual numbers of phytoplankton species at any
one time cannot be known, making testing of methodology for
accuracy impossible. At each stage variability is introduced
into the data. The nature of phytoplankton distribution rarely
matches the conditions demanded by statistical methods but by
careful sampling and analysis the best possible results can be
obtained.

It is often preferable to make counts of several replicate samples than to rely on a count of a single sample.∜

Phytoplankton samples usually need to be examined at more than one magnification. Larger taxa, such as the dinoflagellate *Ceratium* and the diatom *Biddulphia* can be identified at low power and the whole chamber scanned in a few minutes by horizontal or vertical transects. Smaller taxa are identified at high magnification. If immersion objectives are required for identification it is often best to record the position of the cell and return to it after the entire count has been completed. Care must be taken to avoid counting the same cell twice when the cell for instance overlaps the grid or threads in an eyepiece. Normally algae that cross two of the graticule's four edges are included in the count, whereas those that cross the other two are excluded. The edges for each criterion have to be predetermined and adhered to throughout the count.∜(It is recommended that at least two replicate subsamples are counted to increase confidence limits.)

Various methods have been devised to estimate the abundance of nanoplanktonic cells, but none are universally applicable. Picoplankton cannot always be identified with light microscopy, even when living (the problems associated with fixation have been discussed above). The available methods are described by Thomsen (1986) as:

a.) Counts of living cells with a haemacytometer chamber (Guillard, 1978; Booth *et al*, 1982). The advantages are that cells are seen in perfect shape, but the disadvantage is that they cannot survive for long.

b) Counts of cells using an inverted microscope on preserved water samples (Utermöhl, 1958). The disadvantage is that the fixation can distort or even destroy cells.

c) Counts of cells using SEM (scanning electron microscopy), critical point dried. Photosynthetic and non-photosynthetic cells cannot be distinguished.

d) Estimates of cell abundancⁱes from serial dilutions (Throndsen, 1978, 1996). This may underestimate cell densities and favour certain groups of organisms, depending on the culture medium used.

Butterwick *et al* (1982) compared eight methods for estimating the biomass and growth of planktonic algae using the freshwater colonial diatom *Asterionella formosa*. The eight methods were 1) Inverted microscope counts (Utermöhl method, 1958); 2) Electronic Coulter counter; 3) Nephelometry, using a nephelometer to measure amount of light scattered at 90°; 4) In vivo attenuance, measuring attenuance at 680 nm in a double-beam spectrophotometer; 5) In vivo fluorescence of chlorophyll *a*; 6) Reducing capacity; 7) Extracted chlorophyll *a* by spectrophotometry using hot 90% methanol for extraction and 8)

Extracted chlorophyll *a* by fluorometry using hot 90% methanol
for extraction. Direct counting with an inverted microscope
was adopted as a standard method by which the others were
compared. The results obtained showed that the chemical
methods were all relatively slow, taking 50 minutes to 210
minutes. Both the spectrophotometric and fluorometric
produced similar results.

6.2   Cell Counts

Laboratory counters with five or more keys are useful for
counting predominant species. Depending on the purpose of the
investigation, all organisms encountered should be identified
to species, group of species, genus or algal group.

Phytoplankton is counted in algal units i.e. an individual
cell or a colony. The number of cells in a colony is also
counted and recorded, although this can be done by counting a
set number (10 or more) after completion of the scan if this
is more convenient.

It is common practice in dense phytoplankton samples to count
only a fraction of the sample and estimate the total aliquot
count from this.

Whether the entire base, alternate stripes (Utermöhl 1958) or
one or more diameter transects are counted is a matter of
judgement dependent on the nature of the sample. Diameter
transects help to eliminate any bias caused by uneven settling
in the chamber. The ratio of the whole bottom area of a
chamber to one diameter transect is π n/4, where n is the
ratio between diameter and width of the diameter transect.
The total number of cells in the whole chamber is found by
multiplying the cell numbers by this formula.

For very small organisms where high magnification is required,
it is customary to count a number of Whipple fields and
average the result. The number of fields counted should be
adequate to give a total of at least 120 algal units.

6.2.1   Number of Cells to Count

Assuming a Poisson distribution, it is normally considered
that a count of at least 100 cells should be made to give a
reliable cell number estimate to 95% confidence interval of
the estimate within ±20% of the mean value and 400 cells for a
precision of ±10% of the mean (Lund et al 1958).

There may be an advantage in accepting lower precision in
order to reduce the counting time. One short-cut method
involves recording the presence or absence of species in a set
of random fields or subsamples (Legendre and Watt 1972). The
method assumes a Poisson or binomial distribution of cells in
the sample. The density of material or the size of each field

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should be regulated so that the most abundant species is
present about 80% of the time. It is usually sufficient to
count 30 fields.

6.3 Calculating cells per ml or l

Cell numbers obtained from counts in sedimentation chambers or
counting slides can be converted into cells per millilitre or
litre.

Cells/ml = $\dfrac{\text{No of units counted}}{\text{No. of fields}}$ X $\dfrac{\text{area of chamber/or fields}}{\text{sample volume in mls}}$

6.4 Cell Size

Cell numbers alone may not give a totally accurate picture of
the community, particularly as phytoplankton cell sizes can be
between 1 and 2,000µm in diameter and their volume even more
diverse. Lohmann (1908) was the first investigator to
recognise the need for plasma volumes for determining standing
stock of phytoplankton. Cell size is used for taxonomic
purposes as well as for biomass estimations. Cell size can
vary according to the age of the organism and season (Margalef
1963). Large species may be less abundant than smaller
species but their biomass may be higher. In general smaller
forms have a more intensive metabolism and divide faster than
larger forms; they may also be the main producers in a
nutrient rich area.

The calculation of species cell volumes to estimate
phytoplankton community biomass is complicated by the
variations that can occur in both time and space and it is
therefore unwise to rely on measurements taken elsewhere. When
size/volume is thought to be important, a set number of cells
in the sample should be measured and the figure applied to the
cell counts. The effects of fixatives in shrinking cell size
and cell vacuoles should also be borne in mind, for this can
affect plasma volume.

6.5 Serial Dilution Culture (SDC) method

The main purpose of the serial dilution-culture method is to
provide material for identification and enumeration of the
non-preservable fraction of the phytoplankton (the delicate
flagellates). It is based on the assumption that cells will
grow under culture conditions and that cells are evenly
distributed in the sample. From the presence or absence of a
species in different amounts of water the most probable number

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(MPN) per unit can be determined statistically. The principle of the method is to dilute the sample until only one specimen ʋ   x left in the subsample. The number and amount of dilutions enable estimates of the original cell number to be made.

The technique has been used infrequently (Knight-Jones, 1951; Throndsen, 1969). Although it is generally agreed that quantitative estimates obtained by this method are too low it is a practical technique for a quantitative and qualitative survey of the fragile 'naked' flagellates.

The disadvantage of the SDC is its selectivity and dependence on growth conditions; the advantage is that it provides material of delicate organisms, sufficient for identification (Throndsen, 1978, 1996).

6.6 Recommendations for Enumeration

It is important to select an appropriate method for the investigation in hand. The accuracy of any results is dependent on the sampling and subsequent treatment of those samples.

A Summary into a methods + degree of accuracy would be useful

7. CHLOROPHYLL ANALYSIS

7.1 Introduction

Chlorophyll *a* is widely used as a measure of phytoplankton, although it can be a poor estimate of biomass because it assumes algal cells to be homogeneous with respect to their chlorophyll concentration. The variability in chlorophyll content in various species is shown by Madgwick (1966). Chlorophyll degradation products (phaeophytin or phaeophorbide) in sea water samples can be determined by chromatography (Jeffrey, 1974), although this is a lengthy process and unsuitable for routine applications.

The choice of method used for determination of chlorophyll *a* depends to some extent on the level of accuracy required, particularly in respect of degradation products, which have absorption maxima near that of chlorophyll *a*. In field samples a known volume of water is filtered and the pigments extracted from the filter. The results obtained will be on a volume rather than on a cell basis.

The sensitivity of the method described here is adequate except where the chlorophyll content of the water is below $0.2mg/m^3$.

7.2 In-vitro spectrophotometric methods

7.2   In-vitro spectrophotometric methods

Samples are best analysed on the day of collection, or at most after overnight storage in the dark in a refrigerator or cool (4°C) place. Samples should be kept out of strong light and not exposed to high temperatures in transit (Herve and Heinonen 1984). If these criteria cannot be met samples may be frozen on filters, following the filtration step. *— they detuk on the differences in results slow same day ?24 hr. Cooled. Chilled? frozen? Sample?*

Chlorophylls are unstable compounds and very sensitive to light and air, undergoing isomerisations and oxidations. Samples therefore must be subjected to the minimum exposure of light and air, all work should be carried out in dim light and solutions should be kept in glassware, covered with a black cloth.

The volume of water to be analysed will be determined by the local situation and the expected chlorophyll concentration. In addition, factors such as the amount of detritus (which can severely impede filtration) will affect the volume used. As much as 10 litres of seawater can be filtered (Hallegraeff, 1981).

Normally a 1 litre sample of sea water is filtered through a Millipore filter fitted with either a 47 mm diameter Millipore AA filter or a 4.5 Whatman GF/C glass filter paper. Glass filters are cheaper and are recommended if a cell grinding stage is required, to give better extraction. A manostat must be used with glass papers to ensure that the suction never exceeds 0.25 - 0.33 atm as this could cause some pigment to pass through the filter. It was previously standard practice to add magnesium carbonate at the filtering stage in order to prevent phytoplankton chlorophyll becoming acid and decomposing to give phaeophytin pigments. This step is now considered to be unnecessary (Lenz and Fritsch 1980, Lloyd and Tucker 1988). Care should always be taken to ensure that filtration equipment, including filters, centrifuge tubes and spectrophotometer cells are kept free from acid.

Note: if large zooplankters are present these are first removed by straining the sea water through a clean 0.3 mm nylon mesh.

The filter is drained thoroughly under suction before removing it and if necessary cutting away the unstained membrane with clean scissors.

Filters should be extracted as soon as possible, but when this is impractical they can be stored by folding in half, with the plankton inside, and placed in the dark in a desiccator frozen to -20° C. Specimens will keep for a few weeks but such storage usually results in an underestimation of chlorophyll *by how much* levels.

For many years extraction with 90% acetone, or 100% acetone
(Haellegraeff, 1981) was considered satisfactory, although it
was recognised that results were low in many instances because
of the presence of plant cells that were not fully extracted
(Madgwick, 1966; Strickland and Parsons, 1972). In some
species half or more of the pigments were left behind in the
cell and the use of a sonic disintegrator was suggested to
resolve this problem, although the results in improvement do
not merit the application of such equipment on a routine basis
(Strickland and Parsons, 1972). Yentsch and Menzel (1963)
recommended the use of a tissue grinder, which is relatively
convenient but can fail to give complete extraction in a
reasonable time. Other workers have developed specially
designed spectrophotometers to record the diffuse
transmittance spectra of phytoplankton assemblages
concentrated on glass-fibre filters (Faust and Norris, 1985).
This method has the advantage of being quick to perform, the
authors claim analysis takes about 2 minutes per sample, but
tests with other methodologies would be required to assess
accuracy and practicality.

Ethanol is now the preferred solvent for chlorophyll
extraction, in line with European recommendations (see DIN
1986, DS 1986; Marker, 1994). Methanol is permitted as the
second choice but requires greater care in its use (COSHH)
reducing the speed of analysis and is not recommended here.
Acetone extraction is normally used for the separate
measurement of chlorophylls *a*, *b* and *c* and other chloroplast
pigments.

7.2.1   Recommended Procedure: Ethanol Extraction

(i)   Filter a measured volume of sample through a glass-fibre
filter clamped in a suitable holder. For most natural waters 1
litre is a suitable volume but this may need to be adjusted if
the phytoplankton content is abnormally high or low.

(ii)   After filtration is complete reduce the residual water
by allowing air to be drawn through for a short time,
approximately 30 seconds. Discard the filtrate.

(iii)   Remove the filter from the holder and allow to dry in
the dark.

(iv)   Weigh the filter to determine the weight of residual
water.

(v)   Fold the filter three times and transfer to a test tube.

(vi) Follow one of the following methods (a - c):

   a.   Working in a fume cupboard add a known volume of hot
        (70°C) ethanol, sufficient to cover the filter. This
        will usually be 15 or 20 ml.

b. Add a known volume of cold ethanol, usually 15 or 20
    ml, sufficient to cover the filter, and heat to
    boiling by dipping the tube in a water bath held at a
    temperature just above boiling point of ethanol
    (78.5°C). Boil for 2-3 seconds with the tube covered
    to prevent loss of solvent.

c. Add a known volume of cold ethanol, usually 15 or 20
    ml, sufficient to cover the filter. Stopper the tube
    and place in a dark, cold (4°C)., place for 12 hours
    (e.g. overnight). Agitate the filter from time to
    time.

(vii) Add distilled water so that the final concentration is
10% water in ethanol. If 20 ml of 100% ethanol was used
initially, a total of 2.2 ml of water will be needed to make
the final solution 90%, but take into account the residual
water in the filter, which should weigh between 0.5 - 0.7 g
for a 9 cm filter, and adjust the water accordingly.

(viii) Operating in dim light, ensure that the sample is well
mixed, using a vortex stirrer. With forceps, remove the filter
paper from the ethanol and squeeze it against the side of the
tube so that as much ethanol as possible drains back into the
tube.

(ix) Either, centrifuge the extract in a stoppered tube to
obtain a clear extract, or, filter the extract through a small
GF/C filter into a clean tube. Retain the extract in a
stoppered tube, in the dark, for absorbance measurements.

(x) Fill a stoppered spectrophotometer cuvette (generally 10
mm or 40 mm pathlength) with the pigment extract. Let the
pathlength used be d mm. Use 90% v/v aqueous ethanol in the
reference beam of the spectrophotometer.

(xi) Measure the absorbance of the extract at wavelengths of
665 nm and 750 nm. Note that absorbance at 665 nm should fall
within the range of 0.05 to 0.70 units; otherwise adjust
either the volume of the sample, the volume of aqueous
ethanol, or the pathlength of the cell, to meet these
criteria. Absorbance at 750 nm should not exceed 0.005 units
per 10 mm of pathlength, i.e. 0.02 units in a 40 mm pathlength
cell.

(xii) Subtract the absorbance value obtained at 750 nm from that obtained at 665 nm and let this be A. The chlorophyll *a* content of the sample expressed as µg l^{-1} ($\equiv$mg m^3) is obtained from the equation:-

$$\frac{12.2 \times A \times v}{d \times V}$$

Where A = net absorbance
        v = volume of solvent in ml
        V = volume of original filtered sample in litres
        d = cell pathlength in cm
   12.2 = the reciprocal of the specific absorption
            coefficient at 665 nm for chlorophyll *a* in ethanol.

Note: This calculation makes no correction for the presence of degraded matter or other pigments.

7.2.2 Acetone Extraction

This method is recommended only when it is required to separate other pigments in addition to chlorophyll *a*.

(i) Filter the sample as in 7.2.2. above.

(ii) Place the filter in a 15 ml stoppered graduated centrifuge tube. For Millipore filters add approximately 8 ml of 90% acetone and shake the stoppered tube vigorously to dissolve the filter. If a glass paper was used add approximately 10 ml of 90% acetone and shake the stoppered tube vigorously to disintegrate the paper. A "Potter" type grinder may be used to which the glass filter and 2 ml of 90% acetone is added and the grinder run for 1-2 mins in subdued light. After use the pestle should be rinsed into the tube with a few millilitres of 90% acetone and the contents of the grinder tube transferred to a 15 ml centrifuge tube. The total volume in the centrifuge tube should not exceed 10 mls. Allow the pigments to be extracted by placing the tube in a refrigerator in complete darkness for about 20hr.

(iii) Remove the tubes and allow them to reach room temperature, in the dark. Add 90% acetone to make the extracts from Millipore filters up to exactly 10.0 ml and those from glass filters to exactly 12 ml, to allow for the solvent left in the pulp.

(iv) Centrifuge the content of the tubes, with plastic stoppers, for 5 - 10 minutes. In most centrifuges 3000 - 4000 rpm for about 10 minutes is satisfactory. When glass filters have been used tubes should be centrifuged for about 2 minutes and the tubes removed and flicked gently to remove any fibres adhering to the side of the tube. The tubes are then returned to the centrifuge and spun for a further 5 minutes.

(v) Decant the supernatant liquid into a 10 cm path-length spectrophotometer cell designed to hold 10 ml or less of

liquid.

(vi) Without delay measure the extinction of the solution against a cell containing 90% acetone at 750nm and 665nm.

(viii) Calculation of amount of chlorophyll a. Subtract the absorbance value obtained at 750nm from that at 665nm and let this figure be A.

The chlorophyll a content of the sample, expressed as $\mu m \ 1^{-1}$:-

$$\frac{11.9 \times A \times v}{d \times V}$$

Where A = net absorbance
       v = volume of solvent in ml
       V = volume of original filtered sample in litres
       d = cell pathlength in cm
    11.9 = represents a factor derived from the specific absorption coefficient at 665 nm of chlorophyll a in acetone.

7.2.3 Quality Assurance Procedures

It is recommended that tests to check sources of inaccuracy are made regularly.

• Always use matched spectrophotometer cuvettes

• Number these cuvettes on their base and always use them in the same location in the multiple cell holder and always in the same orientation.

• Before using, check the variations between cuvettes by measuring the absorbency at 750 and 665 nm, filled with the standard solvent in normal use. These values will give the extent of cuvette to cuvette differences and must be recorded in a log book. When used regularly, for example every month, changes will indicate deteriorating optical surfaces or accumulated deposits.

• Spectrophotometers nowadays have built-in wavelength checking mechanisms which are activated when they are powered up. Standard didymion filters, which have two sharp absorption maxima at 573 and 585 nm, can be used to check the wavelengths.

• Standard solutions of potassium dichromate ('Spectrosol' for calibration of spectrophotometers obtainable from Merck) can be used to check the absolute accuracy of the absorbency reading.

The absorbency of the extract is partly controlled by the volume filtered, the volume of the extractant and the path length of the cuvette.

7.3 In Vivo Fluorescence

Chlorophyll concentration can also be measured with a fluorometer. The method has a number of advantages over the spectrophotometric technique, such as having a higher sensitivity, requiring less concentrated samples, being fast and direct. A fluorometer emits short wavelength light that is absorbed by chlorophyll molecules and re-emitted (fluoresced) at a longer wavelength. The intensity of the fluorescence is measured by the fluorometer, and this reading can be used to calculate the pigment concentration. Among eucaryotic algae there is considerable difference in the wavelengths of excitation for chlorophyll a fluorescence between organisms with a carotenoid-protein-complex and those without (Yentsch and Yentsch, 1979). The major difference is that the presence of fucoxanthin in diatoms or peridinin in dinoflagellates allows chlorophyll a to be excited efficiently at wavelengths of 525-530 nm in addition to wavelengths of 450 nm.

The method for measuring chlorophyll a and phaeopigment by fluorescence is normally Lorenzen's (1966). Solutions of pure chlorophyll a (obtainable from Sigma Chemical Co) are used to calibrate the fluorometers.

The use of fluorescence allows continuous monitoring and has been used for remotely sensing algal blooms by multiwavelength laser induced fluorescence (Mumola et al., 1975). This method is recommended where sample volumes are restricted or very high inorganic concentrations prevent efficient filtration. It is also the standard method where on-board pumped water systems are used in connection with an auto-analyser for continuous study of nutrients or pollutants. There are commercially available systems (e.g. Aquatracker II from Chelsea Instruments, London, which, when used in accordance with the manufacturer's instructions, gives an estimate of chlorophyll a per volume of water.

Measurements of chlorophyll a fluorescence are made at 680 nm by excitation of the water sample by light of 430-450 nm.

It is essential that regular comparisons are made between the results obtained with this method and by means of spectrophotometry, as described above. Checks should also be made for background fluorescence which can interfere with the result for chlorophyll.

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A comparison of chlorophyll and phaeophytin measurements on
field and laboratory cultures of algae using fluorometry and
spectrophotometry showed that there was little difference in
the results obtained (Axler and Owen, 1994). These authors
showed that differences in filtration and extraction technique
and in frozen storage time did not result in significant
changes in pigment estimates.

7.4   Aerial surveillance

Mapping of phytoplankton biomass by aircraft (including fixed
wing, helicopter and balloon) and satellite sensors has become
an important tool for studying marine biology in recent years
(Gower and Borstad, 1990). Algal chlorophyll and suspended
sediments are two of the major optically active factors
affecting surface water quality (Richie et al). The spectral
signature of chlorophyll in water is well documented and
remote sensing is an established technology for assessing the
variable spatial and temporal densities of chlorophyll and
estimating biomass (Eppley et al., 1985; Gordon and Morel,
1983; Khorram and Cheshire, 1985; Sathyendranath, 1986; Platt
and Sathyendranath, 1988; Campbell and O'Reilly, 1988; Sousa
and Bricaud, 1992; Lavery et al., 1993). There have been
studies on the effects of varying suspended sediment
concentrations on the signature of chlorophyll (Quibell, 1991;
Han et al, 1994).

Aerial surveillance is achieved via satellite or aircraft
mounted monitoring devices. Sunlight penetrating the upper
layers of the water is changed in its intensity and spectral
composition by absorption and scattering caused by dissolved
and suspended matter and the water molecule itself. Increasing
concentrations of chlorophyll in water result in a
progressively greener colouration due to a combination of this
absorption and increased backscatter at 500 to 600 nm. Remote
measurements of the fluorescence of chlorophyll pigments are
seen over a narrow spectral range centred on 685nm.This
visible-wavelength light and can be used to generate coloured
maps showing suspended sediment distribution patterns and the
concentration of the sediment. The horizontal dimensions and
temporal changes in blooms lend themselves to aerial
surveillance and are easier to spot and map than from on board
ship.

One satellite is the Nimbus-7, launched by NASA in November
1978 which carries a Coastal Zone Color Scanner (CZCS). This
is an experimental radiometer designed to examine the bio-
optical properties of the surface layers of the ocean by
measuring reflectances for specific wavebands in the visible
part of the spectrum. Large spatial scale blooms have been

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monitored very effectively by this satellite (Holligan *et al.*
1983a). Scanners are unable to assess the vertical
distribution of chlorophyll but this is compensated for by the
valuable spatial and temporal distributions of surface
distributions that they can provide, and the additional
information showing, for instance, sea-surface temperatures.

Multi-spectral scanners can be used for pollution monitoring,
mapping red tides and can provide information of chlorophyll
concentration. Chlorophyll absorption and fluorescence is
between 650 and 700 nm, with a peak at 685 nm. Different
pigments in some phytoplankton are detectable at different
peaks. Yentsch and Phinney (1990) found that there are three
main fluorescence bands associated with marine micro-
organisms:

(i)   A broad band emission centred around 470-490 nm and
      extending to around 600 nm. Heterotrophic
      dinoflagellates have been shown to exhibit such
      blue-green fluorescence (Mazel 1990).
(ii) An emission band centred around 570-585 nm due to
      phycoerythrin (Yentsch and Phinney 1990)
(iii) The 685 nm emission of chlorophyll *a.*

An enhanced understanding of the results obtained from aerial
surveillance is possible with the examination of water samples
taken from the scanned area at the same time. High reflectance
detected by the CZCS on Nimbus 7 along the continental shelf
edge was found, on examination of water samples, to be due to a
coccolithophorid bloom (Holligan *et al.* 1983b). Comparisons
between chlorophyll levels measured on board ship and by
satellite have been reported by several other authors (Afonin
el al, 1992; Gordon *et al*, 1980, 1983; Smith and Baker, 1982;
Peláez and Guan, 1982 and Guan *et al*, 1985). They have shown
than under cloud-free though generally hazy conditions, an
elaborately processed CZCS image can measure the distribution
of upper-layer chlorophyll for pigment concentrations ranging
from 0.05 to 5.0 mg pigment m^{-3} with an error of 40% or less.

Further use should be made of spectral signatures for
identifying organisms such as *Noctiluca* (Dinophyta),
Coccolithophorids and some bloom-forming diatoms.

7.4.1   NRA's Use of Aerial Surveillance

The NRA's group responsible for aerial surveillance is based
at the National Centre for Instrumentation and Marine
Surveillance, Bath. The National Centre has developed an
aerial survey system utilising the Compact Airborne
Spectrographic Image (CASI). The twin engined aircraft used is
located at an airfield in Coventry and carries out

approximately three complete flights around the British coast   ?
each year.   In addition to being fitted with a CASI (The   Scanner
aircraft has a thermal scanner, colour video and stills
camera. To ensure accurate position referencing and geo-
correction of images, a satellite navigation package has been
integrated into the system. The advantages of CASI over
satellites are:

  · Resolution
  · Adaptability
  · Avoidance of cloud cover
  · Cost and controllability

This aerial survey provides:

  · Monitoring and pollution detection
  · Estimates of chlorophyll *a* for use in eutrophication
    studies
  · Estimates of suspended solids concentration and changes
    in coastal morphology
  · Mapping of mixing zones, outfalls and rivers
  · Identification and tracking of dissimilar bodies of water
  · Land classification

A major limiting factor in the use of aerial surveillance
for phytoplankton monitoring is the weather.

The aerial surveys conducted by the National Centre are linked
to the National Coastal Baseline Survey. This latter programme
detects the major natural changes in the coastal ecosystem on
a seasonal basis. The key elements are:

  · Spot samples about every 15 Km
  · Continuous monitoring
  · Entire coverage with airborne imagery
  · Co-ordination of survey between aircraft and boats
  · Standardised methodologies and chemical analysis

It is recommended that there is greater liaison between the
aerial surveillance team and the shore/ship based sampling
teams. This would assist both sides to obtain the maximum
information from their efforts and help the planning of future
sampling strategies.

Further work should be carried out to assess the value of
certain bandwidths to enable specific identifications of bloom
algae. Some have been measured, for example, diatoms(?) and   X
the dinoflagellate *Noctiluca scintillans* (Macartney) Ehrenb.
(Holligan *et al.*, 1983a; Balch and Haxo, 1984) in the aerial
surveillance data.

The National Centre is the NRA's specialist point for all
remotely sensed applications, including satellite data.
Further information regarding the availability of aerial
surveillance may be obtained from:

> NRA National Centre for Instrumentation & Marine
> Surveillance,
> Rivers House,
> Lower Bristol Road,
> Bath, BA2 9ES
>
> Telephone: 01278 457333

7.5   Chlorophyll *a* and Cell Counts

Comparisons and correlations between cell counts and
chlorophyll *a* levels should be made to build up an
understanding of the environment and for potential model
building.   It has been estimated that 4µg of chlorophyll *a* are
approximately equivalent to 1 cm^3 of cell volume (Bailly-Watts
quoted in NRA Freshwater Phytoplankton Standard Methodologies,
1993). If the chlorophyll *a* result is significantly greater
than the combined cell volumes an error exists. It should be
borne in mind that computation of cell volumes is not without
its difficulties and sources of error.

The dominant taxa responsible for chlorophyll *a* peaks can be
determined by examination of cell counts only if straight
water samples have been counted and that a bias has not been
introduced, such as loss of smaller plankton by filtration or
fixation.

8.   DATA HANDLING AND ANALYSIS

8.1   Introduction

There is no internationally agreed format for the exchange of
phytoplankton data, nor any agreed system of numerical labels
for species or other taxonomic categories (Colebrook in:
Sournia 1978). The objective of a data storage system is to
facilitate retrieval and processing.   The format and structure
of the stored data is determined by the requirements of the
retrieval procedures.

Computer database systems are now more user friendly and allow
for simple inputting of data, and mathematical manipulation.
Robson and Bailey-Watts (1978) describe a system for recording
and sorting plankton data.

The sampling and subsampling design, including numbers and volumes of each subsampling level, should be recorded so that comparison with data from other designs can be made.

Where possible all data, especially long term monitoring data, should be kept in a suitable database. Data entry should be simple, logical and include an error correction facility. The database should allow easy sorting, searching and retrieval of data and should be able to produce simple time course plots of individual, or groups of taxa, or interface with a suitable spreadsheet. The database can either contain the bare minimum of information to allow searches, and be supported by a secondary system of fuller information (digital and/or hard copy), or hold all relevant information in one. If a combined system is used it should interface with an entry program which permits simultaneous data entry to both systems.

Each database file should hold enough records to facilitate the maximum number of taxa encountered per sample.

Whatever database design is adopted it should always be possible to work back to the raw data.
It is not sufficient to just maintain a database of final results which cannot be referenced or cross-checked against the original notebook or data sheet. The requirement for adequate backup of data cannot be overstressed.

Reference should be made to the NRA Freshwater Phytoplankton manual, pp 64-67, for details of the system in use by the NRA for freshwater algae, which is entirely appropriate to be extended for use with marine phytoplankton.

8.2   Data Handling by the NRA

The Marine Section of NRA's Anglian Region produces coastal reports from the water quality monitoring of its vessel 'Sea Vigil'. Field data, information on nutrients gathered from the on-board autoanalyser are entered onto the laboratory database at the Anglian Region's Headquarters in Peterborough.   The data is presented as a mixture of graphs and charts, by each survey showing:

- Table of Data
- Chart of Sample Programme, showing Order of Sampling
- Graph of Nutrient Data Values against Site
- Contour Plot of Ammonia Data
- Contour Plot of Phosphate Data
- Contour Plot of Silicate Data
- Contour Plot of Nitrate Data
- Contour Plot of Nitrite Data
- Contour Plot of Chlorophyll *a* Data
- Contour Plot of Temperature Data
- Contour Plot of Salinity Data
- Contour Plot of Dissolved Oxygen Data.

The ecological value of being able to add phytoplankton and zooplankton data to the above information would be immense.

The NRA's National Centre for Instrumentation and Marine Surveillance operates data-handling systems. The data collected by the CASI and thermal scanner is rapidly processed to a very high standard using image processing techniques. These images can be fitted to a map so that they can be cross indexed and calibrated with data gathered from the ground or water. Images are processed to give valuable results such as chlorophyll quantities and suspended sediments.

The survey data in all its forms is collated into a series of archives and databases. The databases are tailored to each data type, allowing rapid searching and retrieval of data into either electronic or hard copy. Routine outputs from the surveys are sent to the regions, as well as national assessments to the Head Office.

8.3 Taxa Codes

For the sake of simplicity, taxa are normally stored within a database as an individual code. Many coding systems are available, all with their own limitations, and the one recommended was developed by Whitton, Diaz and Holmes (1979). Although this coding system requires updating, with the possible upgrading from a six to eight digit code to accommodate extra taxa, in most cases it can be used without modification.

The system uses a six digit number, in which the first two are the Phylum Pair, the second two the Genus Pair and the remaining two the Species Pair. This makes it possible to search at different taxonomic levels. As many of the species pairs are unused, there is scope for the use of size classes for unidentified taxa. For example 'small centric diatoms <5µm in diameter'. Since the development of this system many more species have been described within some genera, and the present species pairs will probably require expansion to a four digit field to accommodate them (Whitton, pers. comm.).

9. TAXONOMY

A list of references for taxonomic purposes is contained in Appendix III and training in this area is covered in the Training Section below.

Taxonomy is an integral component of enumeration; a taxon cannot be counted until it is identified as being discrete. This does not mean that each taxon encountered has to be given its full generic and specific name, but some sort of 'label' must be given to it for numerical purposes. If the

unidentified taxon constitutes a significant part of the
phytoplankton population it may be necessary to have it
identified by an expert, either internal or external, for
instance at the Natural History Museum, (London), which has an
extensive collection of mounted material. A careful
description should be made, including a labelled drawing and
if the equipment is available a photograph should be taken.

Some species which are known to cause nuisance or blooms are
listed below.

Gyrodinium aureolum Hulburt - produces blooms which are
important for their primary productivity (Holligan, 1981) and
can cause extensive animal mortalities (Tangen, 1977; Ottway
et al, 1979; Forster, 1979)

Noctiluca

Phaeocystis

Alexandrium tamarense

10. TRAINING

Training is an important feature of a quality system. Not only
is it essential for beginners, but it is necessary for
established staff to be informed of new developments, both in
their own region and in other regions of the NRA and Europe.

Where possible, training should be given by experienced staff.
Safety and quality of work should be given special attention.
Identification of species requires an introduction to taxa,
help when required and if possible reference slide,
photographs and drawings of species likely to be encountered.
If experienced inhouse staff are unavailable training days
should be organised using outside experts. A list of marine
phytoplankton specialists (with their particular area of
expertise indicated) who run such courses is given below:

Cox, Dr. E. (Diatoms) The Natural History Museum, Cromwell
                      Road, London SW7 5BD
                      (0171 938 9001)

Dodge, Prof. J.D. (Dinoflagellates) Royal Holloway College,
                      Egham, Surrey, TW20 0EX
                      (01784 443774)

Green, Dr. J.C. (Haptophytes and Prasinophytes) Plymouth
                      Marine Laboratory, Citadel Hill,
                      Plymouth, PL1 2PD
                      (01752 222772)

Lewis, Dr. Jane (Dinoflagellates) University of Westminster,
                    115 New Cavendish Street, London W1M 8JS
                    (0171 911 5000)

Lucas, Dr. I.A.N. (Cryptophytes) School of Ocean Sciences,
                    University of Wales, Menai Bridge,
                    Gwynedd, LL59 5EY
                    (01248 351151)

11. RECOMMENDATIONS

It is recommended that procedures are monitored and adapted,
if necessary, to accommodate new technologies, and changes in
ecological conditions.
To be expanded

The recommended methods for marine phytoplankton are detailed
in the Biology Laboratory Procedures Manual, Marine
Phytoplankton Methodology, NRA, Anglian Region, 1996.

APPENDICES

Appendix I

Experts contacted in connection with this report
(All located at Kingfisher House, Peterborough unless stated
otherwise)

Chris Ashcroft, Senior Marine Survey Officer

Dave Balbi, Biology Technician (Northern Area), Spalding

Brian Barnett, Marine Scientist (Northern Area), Lincoln

Robin Chatterjee, Eutrophication Scientist, Toxic and
Persistent Substances Centre (TAPS)

Alison Frogley, Toxic Algae Scientist (TAPS)

Terry Clough, Senior Biologist (Central Area), Brampton

Alastair Ferguson, Regional Biologist

David Foster, Directives Scientist

Barrie Harbott, Regional Water Quality Planner

Mark Johnson, Marine Scientist (Eastern Area), Ipswich

Richard Saull, Data Manager, Twerton

Robert Davidson, Twerton

John Nicols, MAFF, Lowestoft

Richard Thompson, Port Erin Marine Laboratory, Isle of Man

Dr E. Cox (Diatoms), The Natural History Museum, London

Dr. J.C. Green (Haptophytes and Prasinophytes), Plymouth
Marine Laboratory

Dr Jane Lewis (Dinoflagellates) University of Westminster

Dr I.A.N. Lucas (Cryptophytes), School of Ocean Sciences,
University of Wales

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APPENDIX III
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