EU Working Group on the Microbiological Monitoring of Bivalve Mollusc Harvesting Areas

A Review of Scientific Information and Current Monitoring Practices in EU Member States and Elsewhere

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Executive Summary

It is recognised that the consumption of live bivalve molluscs involves a risk of infectious illness due largely to sewage-derived pathogens. Legislation within the EU involves an assessment of such risk of each harvesting area based on monitoring with faecal indicator bacteria. Such an assessment leads to a classification for the area which dictates the level of post-harvest treatment to which the bivalves must be subjected before sale for consumption.

There are several components to a microbiological monitoring programme and these include:

- The content of sampling plans (where samples are taken, how often, under what conditions)
- Sampling methods and sample transport protocols
- Comparability of test methods
- The numerical standards that are applied
- Interpretation of monitoring programme data in relation to these standards

Variation in the application of these components could affect the uniformity of both public health protection and trade constraints across the EU. The network of European reference laboratories for monitoring viral and bacteriological contamination of bivalve molluscs previously expressed concern that this was the case and proposed that an expert working group be established to produce a good practice guide to help address this. This document reviews the scientific evidence relating to the construction of microbiological monitoring programmes for bivalve molluscan fisheries and the approaches taken within Europe and elsewhere to the application of these.

The outcome of this work supports the concern expressed by the reference laboratory network and resulted in the following recommendations:

1. The Working Group proceeds with the drafting of the Good Practice Guide based on the information received during the preparation of this review.
2. The contents of the guide should be based as far as possible on the principles of good public health protection and scientific information.
3. Additional research needs should be identified in order to improve the information available when the Guide is revised in future. This research will then need to be supported by the Commission and the Member States. Areas for research would include:
   i. Sanitary surveys: identification of practical approaches to determining the essential elements for each harvesting area
   ii. Relative spatial, temporal and species–specific variation in *E. coli* and pathogens using quantitative methods (Norovirus and Hepatitis A as viral pathogens; *Salmonella* as a bacterial pathogen).
   iii. Studies to support sampling and transport protocols
   iv. Methods for the analysis of monitoring data for the purposes of classification
4. All Member States should ensure that they have written procedures for the components of their monitoring programmes such as selection of monitoring points, sampling and sample transport, laboratory analyses and interpretation of data.
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Microbiological monitoring of bivalve mollusc harvesting areas: review of scientific information and current practices in EU Member States and elsewhere

1. General introduction

Many of the estuaries and coastal areas used for bivalve mollusc cultivation and harvesting in Europe are also used for sewage disposal. Bivalve molluscs obtain their nutrients by sieving particles from the water column and thus any contaminants in the water are concentrated and retained. Bivalve molluscan shellfish are often eaten raw or lightly cooked and so there is not a final opportunity to kill the pathogens before consumption. Outbreaks of disease can occur on an epidemic scale. Food safety concerns regarding the consumption of sewage-contaminated bivalve molluscs, particularly with regard to their role in outbreaks of typhoid, were expressed as far back as the 1890’s (Buchan 1910). Large outbreaks of typhoid associated with the consumption of contaminated bivalve mussels during the early part of the 20th century led to the establishment of national controls in both the Europe and the US. National controls within Europe were superseded by the implementation of the Directive 91/492/EEC (Shellfish Hygiene Directive; European Communities 1991). The microbial illnesses primarily associated with bivalve mollusc consumption are predominantly due to Noroviruses although in some countries Hepatitis A and bacterial illnesses, such as Salmonellosis and Campylobacteriosis, also occur (Rippey 1994; Lees 2000).

Classification of live bivalve mollusc (LBM) production areas is undertaken within the EU, and certain other countries having agreements with the EU, under Directive 91/492/EEC. The classification is based on monitoring for faecal coliforms and/or \textit{Escherichia coli} in bivalve mollusc flesh and intravalvular liquid (FIL), and yields an assessment of risk of contamination of LBMs with pathogens and dictates the level of treatment to which the bivalves must be subjected prior to sale for consumption. The levels are given in Table 1.1. There are also requirements for the ongoing monitoring of production areas by the competent authority. The consolidated food hygiene official control regulation (Hygiene 3) contains similar, although not identical, requirements and these are given in Table 1.2 (European Communities 2004a). The new regulations also contain new requirements for sanitary surveys (see Section 2) and for the classification, where appropriate, of production areas for wild \textit{Pectinidae}. The latter are currently completely exempt from the need for classification.

In the European legislation, the classification and monitoring requirements apply to both production and relaying areas. In this document, the term harvesting area will be used to cover both situations.

Table 1.3 gives an overview of the number of classified areas within each Member State or (semi-)autonomous region.

General properties of a microbiological indicator of faecal contamination

A number of different authors have defined the requirements for an ideal indicator organism. The following are after Godfree \textit{et al}, 1997.
• Occurs exclusively and consistently in human or animal faeces
• Is present in greater numbers than the pathogens
• Does not multiply in the environment
• Is capable of easy detection using simple and reproducible methods
• Of similar environmental resistance to the pathogens
• Of similar ecology to that of the pathogen

Coliforms: Coliforms are oxidase-negative Gram-negative bacteria that ferment lactose at 37°C with the production of gas. They have a long history of use as an indicator of contamination of water and food. They may arise from a large variety of sources, including sewage, soil and vegetation.

Faecal coliforms: This is a subgroup of coliforms that has a long history of use as an indicator of faecal contamination. These organisms are selected by incubating an inoculum derived from a coliform enrichment broth at higher temperatures (44°C – 45.5°C). Thus, the group of faecal coliforms has a higher probability of containing organisms of faecal origin and hence indicating faecal contamination than the coliform group as a whole. However, some of the bacterial species that are included in the faecal coliform group may be associated with soil and/or vegetation and thus may still be derived from non-faecal sources of contamination. Faecal coliforms, including E. coli, will not multiply in live bivalve molluscs stored at 10°C or less for several days (Cook & Ruple 1989).

E. coli: The natural habitat for this organism is the intestines of human and vertebrate animals. In temperate waters this organism is absent from fish and crustaceans at the time of capture (except in grossly polluted waters). Moreover, fish and shellfish should always be held at temperatures below those, which support growth. This organism is therefore particularly useful as indicator of contamination (small numbers) or mishandling such as temperature abuse in product handling (large numbers). Contamination of food with E. coli implies a risk that one or more of enteric pathogens may have gained access to the food. However, failure to detect E. coli does not assure the absence of enteric pathogens (Mossel 1967, Silliker and Gabis 1976). E. coli does not multiply in the marine environment in temperate climates but it is known that E. coli, as well as some other bacterial indicators, will survive, and even multiply, in both the terrestrial and marine environments in subtropical and tropical climates (Byappanahalli & Fujioka 1998; Bordalo et al. 2002).

The resistance of E. coli to adverse physical and chemical conditions is low. This makes E. coli less useful as an indicator organism in examination of water as its numbers will be reduced over a period of hours due to the effect of ultraviolet light, osmotic stresses and protozoal predation. Thus it is well established that enteric viruses survive much longer than E. coli in seawater (Melnick and Gerba 1980). Another complicating factor is that viruses are removed much more slowly (days to weeks) from bivalve molluscs when exposed to clean seawater than is E. coli (hours). Given that the intent of the legislation is to protect the consumer from exposure to the pathogens, and not the bacterial indicators, these factors need to be kept in mind when designing and implementing monitoring programmes.

The requirements of the Shellfish Hygiene Directive are relatively vague, certainly compared to those given in some other countries (e.g. the US National Shellfish Sanitation Programme (NSSP)), and approaches to the microbiological monitoring of harvesting areas vary greatly between different Member States. Some of the differences are valid, reflecting local considerations, while others greatly affect the consistent application of public health controls.
within the EU, with consequent effects on the equivalence of trade constraints. Discussions at the annual workshops of the National Reference Laboratories for monitoring bacteriological and viral contamination of bivalve molluscs resulted in recommendations that a working group be formed to consider the matter and to propose a Good Practice Guide for the Microbiological Monitoring of Bivalve Mollusc Harvesting Areas.

Reference laboratory network workshop 2002:

10. The NRLs agreed that a working group should be established to produce a guidance document for the microbiological monitoring of shellfish harvesting areas.

11. Further to the preparation of the guidance document the NRLs recommended that certain criteria relating to the microbiological monitoring of shellfish harvesting areas inter alia, sample size, sample frequency, conditions of transport, should be specified in the relevant Community legislation.

Reference laboratory network workshop 2003:

24. Discussion on the official controls proposal (hygiene 3) highlighted several areas which would benefit from more detailed technical discussion. This included the numerical standards for faecal indicator analysis and the analytical tolerance applied, sampling methods and plans, and the requirements for detailing polluting influences in production areas. NRLs considered that a working group should consider these aspects and make recommendations/guidance as appropriate.

25. Further to the above the CRL agreed to propose an agenda and make recommendations to the Commission. NRLs agreed to subsequently propose possible working group members with details of their expertise.

26. Further to the above the NRLs agreed to supply relevant documents on practices for classification of production areas to the CRL to inform members of the working group.

Reference laboratory network workshop 2004:

23. NRLs recognized the importance of the Microbiological Monitoring Working Group in developing a best practice guideline and were invited to provide information on classification practices and data handling in their Member States to the WG to inform its discussion. The WG particularly requested scientific data supporting such practices. The WG would feedback to NRLs at the next annual workshop.
The purpose of the present review is to provide background information towards the development of that good practice guide. The content of the review will largely be based on the present and impending requirements of European legislation, although analogous requirements of other systems will also be examined. This means that the risk assessment aspects in general, including microbiological monitoring, will concentrate on those related to faecal contamination. Contamination of harvesting areas with autochthonous marine bacteria which may be pathogenic to humans, such as *Vibrio parahaemolyticus* and *Vibrio vulnificus*, will not be considered. Some of the aspects relevant to these organisms have been reviewed at both the international and European levels (FAO/WHO 2001, 2002; SCVMPH 2001) and the role of controls are being considered at Codex (Codex 2003).

The review will consider the following aspects:

a) Sanitary surveys  
b) Sampling plans  
c) Sampling and sample transport  
d) Numerical standards and analytical tolerance  
e) Data handling and storage  
f) Interpretation of data

and will contain scientific consideration of these aspects together with an appraisal of practices in those countries with bivalve mollusc harvesting areas that were Member States of the EU prior to 1 April 2004. Where approaches differ significantly between regions of a Member State, the relevant information will be presented where available. Fuller details of the legislative requirements, both under Directive 91/492 and the Hygiene 3 Regulation, will be given in the relevant sections of the review.
Table 1.1. Criteria for the classification of bivalve mollusc harvesting areas under Directive 91/492

<table>
<thead>
<tr>
<th>Classification</th>
<th>Microbiological standard per 100g bivalve mollusc flesh and intravalvular fluid</th>
<th>Treatment required</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>All samples &lt;230 <em>E. coli</em> or &lt;300 faecal coliforms</td>
<td>None</td>
</tr>
<tr>
<td>B</td>
<td>90% of samples &lt;4,600 <em>E. coli</em> or &lt;6,000 faecal coliforms</td>
<td>Purification, relaying in class A area or cooking by an approved method</td>
</tr>
<tr>
<td>C</td>
<td>All samples &lt;60,000 faecal coliforms</td>
<td>Relaying for at least 2 months, intensive purification, or cooking by an approved method</td>
</tr>
<tr>
<td>Prohibited</td>
<td>&gt;60,000 faecal coliforms</td>
<td>Harvesting not permitted</td>
</tr>
</tbody>
</table>

Notes
1 Some EU Member States have incorporated an equivalent *E. coli* value of 46,000 per 100g into national legislation (France; UK).
2 Intensive purification has not been defined as required under the Directive and therefore this option does not exist in practice.
3 The competent authority may prohibit any production and harvesting of bivalve molluscs in areas considered unsuitable for these activities for health reasons.

Table 1.2. Criteria for the classification of bivalve mollusc harvesting areas under the Hygiene 3 Regulation

<table>
<thead>
<tr>
<th>Classification</th>
<th>Microbiological standard per 100g shellfish flesh and intravalvular fluid</th>
<th>Treatment required</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>All samples &lt;230 <em>E. coli</em></td>
<td>None</td>
</tr>
<tr>
<td>B</td>
<td>All samples &lt;4600 <em>E. coli</em></td>
<td>Purification, relaying in class A area or cooking by an approved method</td>
</tr>
<tr>
<td>C</td>
<td>All samples &lt;46000 <em>E. coli</em></td>
<td>Relaying for a long period or cooking by an approved method</td>
</tr>
<tr>
<td>Prohibited</td>
<td>&gt;46000 <em>E. coli</em></td>
<td>Harvesting not permitted</td>
</tr>
</tbody>
</table>

Notes
1 The competent authority may prohibit any production and harvesting of bivalve molluscs in areas considered unsuitable for these activities for health reasons.
Table 1.3  Overview of the total numbers of production areas and the number classified as A, B C (and D) if information available.

<table>
<thead>
<tr>
<th></th>
<th>Total number of production areas</th>
<th>Total number of aquaculture establishments</th>
<th>Number of A classifications</th>
<th>Number of B classifications</th>
<th>Number of C classifications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belgium</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Denmark</td>
<td>71</td>
<td>4 to be active in the near future and several under development</td>
<td>137 zones in 2002 91 zones in 2001</td>
<td>71</td>
<td>0</td>
</tr>
<tr>
<td>France</td>
<td>460 zones within which approximately 54200 parcels are placed along the coastline (provisional classification: 31 zones)</td>
<td>3850 expedition establishments</td>
<td>149</td>
<td>222</td>
<td>58</td>
</tr>
<tr>
<td>Germany</td>
<td>No information</td>
<td>No information</td>
<td>No information</td>
<td>No information</td>
<td>No information</td>
</tr>
<tr>
<td>Greece</td>
<td>24</td>
<td>193</td>
<td>22</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Ireland</td>
<td>66</td>
<td>No information</td>
<td>28</td>
<td>36</td>
<td>2</td>
</tr>
<tr>
<td>Italy</td>
<td>The zone included from shoreline to 500 mt off-shore in general is class B or prohibited (in some regions, for striped clams, this limit is 3 mt of depth). During the bathing season, some of the class B shoreline areas are closed. Long-line productions (off-shore) are class A. All lagoon areas are class B.</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Netherlands</td>
<td>19</td>
<td>No information</td>
<td>19</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Portugal</td>
<td>48</td>
<td>39</td>
<td>8</td>
<td>33</td>
<td>7</td>
</tr>
<tr>
<td>Country</td>
<td>Total number of production areas</td>
<td>Total number of aquaculture establishments</td>
<td>Number of A classifications</td>
<td>Number of B classifications</td>
<td>Number of C classifications</td>
</tr>
<tr>
<td>-------------</td>
<td>----------------------------------</td>
<td>--------------------------------------------</td>
<td>----------------------------</td>
<td>----------------------------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td>Sweden</td>
<td>No information</td>
<td>No information</td>
<td>No information</td>
<td>No information</td>
<td>No information</td>
</tr>
</tbody>
</table>

Note: \(^1\) includes 54 class A sites and 98 seasonal class A sites (class B outside the class A season)
2. Sanitary surveys

2.1 Introduction

Table 2.1 gives the sources that may give rise to faecal contamination of bivalve mollusc harvesting areas. The sources of greatest impact will differ from area to area, depending on the relative contributions of the sources in that area, the compounding effect of rainfall on the contribution from the individual sources (such as effectiveness of sewage treatment processes, discharges from combined sewer and surface water overflows, river flows, farming activities, direct land run-off) and the geographical proximity of the source(s) and harvesting areas. The way that tides and currents take the contamination from the source to those areas, and the effect of other environmental factors such as season, temperature, sunshine and wind, will alter the magnitude of the contamination from any one source.

In most examples of contaminating sources given in Table 2.1, the level of risk associated with each source is an expert opinion as formal risk assessments have not been undertaken for such sources with respect to health risks arising from bivalve mollusc consumption. While there is a general view that the health risks associated with human sources (particularly those with large volume discharges) may be of greatest risk, there is a general acceptance that because animal wastes may contain micro-organisms pathogenic to humans (e.g. *E. coli* O157, *Salmonella*, *Campylobacter*, *Cryptosporidium*, *Giardia*), there is currently insufficient evidence to treat contamination arising from different sources any differently in terms of statutory controls. It is also the case that very small discharges (e.g. septic tanks or sewage soakaways) have given rise to outbreaks of illness associated with bivalve mollusc consumption.

It is possible to site aquaculture operations away from gross sources of contamination, or to move existing ones to less contaminated areas. For example, in France, the Regulations prohibit the establishment of such operations in anything but class A or B areas.

2.2 Geographical location and hydrodynamic effects

Determination of the possible impact of contaminating sources initially requires the accumulation of data on all such sources in the area. Primary assessment of such impacts almost invariably requires the plotting of the location of such impacting sources on maps (hard copy or electronic form), together with the location and extent of the harvesting areas. The assessment of impact from each source ideally requires a knowledge of the microbial content and volume of each, together with an assessment of the hydrodynamic effects in the area. The latter may be determined by a range of methods of differing complexity: visual inspection of hydrographic charts and tide tables; use of tidal software programmes; use of dye or microbial tracers; mathematical modelling. Lee and Morgan (2003) showed that significant differences in contamination may be seen under differing tidal states (spring/neap or high/low).

Significant contamination have been reported in harvesting areas up to 7 km from a sewage outfall in a coastal area and it may be that large discharges will have effects extending beyond this distance (Lee & Glover 1998). Dilution and dispersion of the sewage discharge in the receiving water will tend to reduce the concentration of micro-organisms with distance and time. For harvesting areas located in estuaries or rias, contaminating sources located a significant distance inland may have a detectable impact. In the latter cases, it may be that
the contamination contributed by the river at the tidal limit (or the mouth for coastal harvesting areas) is assessed as a single source instead of considering the contribution made by all sources in the catchment of the river (Tattersall et al. 2003). Belliaeff and Cochard (1995) demonstrated significant spatial variation in faecal contamination across a harvesting area and this effect needs to be taken into account when assessing the impact of different sources.

**Table 2.1. Sources of faecal contamination of bivalve mollusc harvesting areas**

<table>
<thead>
<tr>
<th>Source</th>
<th>Level of risk to public health</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>a. Point Source Discharges</strong></td>
<td></td>
</tr>
<tr>
<td>Private/municipal sewage plant</td>
<td>Most significant risk because of diverse contributing population and volume; type of treatment important</td>
</tr>
<tr>
<td>Industrial waste sources (meat processing plants, etc)</td>
<td>Significant risk if wastes involve pathogens capable of causing human disease, or chemicals which can be bio-accumulated; important primarily because of volume of wastes</td>
</tr>
<tr>
<td>Combined sewer overflows</td>
<td>Significant risk because of untreated human waste contribution and volume</td>
</tr>
<tr>
<td>Animal feedlots/poultry houses</td>
<td>Potential human risk because of large aggregation of animals and ability of some domestic animals (pigs, fowl, cattle) to transmit human diseases</td>
</tr>
<tr>
<td><strong>b. Non-point Source Discharges</strong></td>
<td></td>
</tr>
<tr>
<td>Waste discharges from boats</td>
<td>Potential risk due to possible intermittent discharge of small quantities of raw sewage</td>
</tr>
<tr>
<td>Storm drains, street runoff</td>
<td>Potential risk because of some potential for human sewage discharge; risk significantly less than with combined sewers</td>
</tr>
<tr>
<td>Rural land with domestic animals</td>
<td>Significantly less risk (farms, pastures, etc) than direct human sources</td>
</tr>
<tr>
<td>Forest, marsh, etc (dominated by wild animals and birds)</td>
<td>Significantly less risk than human sources on present evidence</td>
</tr>
</tbody>
</table>

(Adapted from Garreis, 1994)
2.3 Effect of environmental factors

There are four main ways that environmental factors can affect the level of faecal contamination that occurs in the bivalve molluscs in the harvesting areas:

a) by altering the amount of micro-organisms discharged into the environment;
b) by altering (usually reducing) the concentration of micro-organisms in the seawater;
c) by altering the way that tides and currents take the contamination to the harvesting area;
d) by altering the uptake and retention of micro-organisms by the bivalve molluscs.

The first group includes season, air temperature and rainfall. Seasonal effects include the impact of tourism increasing the loading of sewage to treatment plants and differences in extent and route of contamination by farm animals. Air temperature will affect survival of micro-organisms on land and also the efficiency of biological sewage treatment processes. Increased rainfall tends to reduce the efficiency of biological sewage treatment processes and to increase the amount of contamination arising from combined sewer overflows, surface water overflows and land run-off. Sedimented micro-organisms, absorbed to particulate matter, may be resuspended during periods of heavy rainfall. Rainfall effects may be determined directly, by association with increased river flows, or by association with decreased salinity levels.

The second group includes the action of sunlight, seawater temperature and salinity. The ultraviolet rays in sunlight tend to kill micro-organisms, but this only has an effect on surface layers, not below 2.5 metres. The turbidity of the water is important and can result in the blocking of up to 50% of the solar radiation. The effect of sunlight tends to vary with both geographical location (being increased towards the tropics), season (being greater during summer) and weather (being greater during cloud-free days). Cold temperatures can prolong the survival of micro-organisms in estuarine and marine environments: this may be partly due to effects on the microbes themselves, but may also be due to lower rates of predation by plankton at lower temperatures. However, at high temperatures, such as those found in the tropics and subtropics, faecal coliforms, including *E. coli*, may actively grow in the environment. Coliform organisms do not survive for extended periods in full salinity seawater and this may reduce the impact determined from distant contaminating sources by monitoring programmes. The presence of organic materials, particularly associated with particulate matter, may extend the time that bacteria survive in the marine environment (Gerba & McLeod 1976). Sedimentation around crude discharges may increase the effect of contamination on bivalves growing on or in the sea bed while the low density of secondary-treated or rainfall-associated discharges may result in increased contamination at the surface. Survival of *E. coli* in the marine environment depends on all these, and possibly other, factors and the $T_{90}$ values (time to 90% reduction) used in hydrodynamic modelling vary from as short as 2 hours to 48 hours or more, depending on daylight and other conditions. Conversely, human pathogenic viruses may survive for periods of weeks. In modelling the effect of a sewage discharge on a harvesting area, Pommepuy *et al.* (2004) used a $T_{90}$ of 30 days for Norovirus.

The third group includes river flows, wind driven currents, together with thermoclines and density gradients, which may modify the basal current systems to affect the way that
contamination impacts on the harvesting area. These effects mean that simple two-dimensional hydrodynamic modelling may not adequately represent the practical situation.

The fourth group includes seawater temperature, salinity and suspended material (Jorgensen 1990). Rates of pumping, and thus of both accumulation and depuration of micro-organisms increase with increasing temperature and the resulting final concentrations in the bivalves will be a complex interrelationship of the two (and may be affected by season). Depuration of faecal bacterial indicators tends to occur more quickly (in a matter of hours) than does depuration of viruses which may take days or even weeks. Salinities significantly higher or lower than the optimum will reduce the pumping action of the bivalves, with the animals ceasing activity and closing at extremely high or low levels. The rate of pumping action, and accumulation of contaminants, tends to increase with the concentration of particulate material up to a certain point, above which it decreases and finally stops. Different bivalve species respond to these factors to different extents.

2.4 European Legislation

Chapter VI of the Annex to Directive 91/492 identifies that sampling plans for the periodic monitoring of live bivalve mollusc relaying and production areas must take into account likely variations in faecal contamination at each production and relaying area. This implies that the likely sources of faecal contamination should be known, how the sources themselves vary in their degree of contamination, and how the impact of such contamination on the bivalve molluscs also varies. However, this is not explicitly stated and no detail is given on the type of information that should be gathered or how it should be used.

The Hygiene 3 Regulations, to be implemented from 2006, give more detail. These state that:

6. If the competent authority decides in principle to classify a production or relaying area, it must:
(a) make an inventory of the sources of pollution of human or animal origin likely to be a source of contamination for the production area;
(b) examine the quantities of organic pollutants which are released during the different periods of the year, according to the seasonal variations of both human and animal populations in the catchment area, rainfall readings, waste water treatment, etc.;
(c) determine the characteristics of the circulation of pollutants by virtue of current patterns, bathymetry and the tidal cycle in the production area; and
(d) establish a sampling programme of bivalve molluscs in the production area which is based on the examination of established data, and with a number of samples, a geographical distribution of the sampling points and a sampling frequency which must ensure that the results of the analysis are as representative as possible for the area considered.

These requirements are more detailed but still allow considerable latitude as to the exact nature and extent of the information to be collected and how it should subsequently be interpreted and utilized. There is still the additional general requirement that sampling plans must take into account likely variations in faecal contamination.

The collection and analysis of information for the purpose of the development of sampling plans and the determination of classifications is termed a sanitary survey. This section of the review will examine:
i) how this issue has been approached to date in EU MSs with bivalve mollusc harvesting areas, taking into account the very general implications of the wording of Directive 91/492.

ii) how this issue has been approached in countries outside the EU which have more explicit requirements for sanitary surveys.

2.5 Approaches taken in EU Member States

2.5.1 Assessing the influence of sources of contamination

A summary of some of the main points of the approaches taken in EU Member States is shown in Table 2.2. Most MS record the position and extent of bivalve mollusc harvesting areas on maps (hard copy or electronic (GIS)) and plot on these maps the position of known sewage discharges and other impacting sources. These are then taken into account when determining the number and position of sampling sites. There is not usually a written protocol for the way this data for a harvesting area is recorded or interpreted. There is also not usually a separate record, with conclusions, for each harvesting area.

However, the French Regulations (Anon. 1999) explicitly state that:

Art 3. The production zones are defined by precise geographical limits by connecting lines to the coast and, each time that is necessary, towards the open sea. These constitute coherent entities. To define these, the following are taken into particular consideration:
- their hydrological characteristics;
- the homogeneity, known or presumed, of their sanitary quality;
- the technical and socio-economic characteristics of the production activities;
- their conditions of access and location

Mathematical models simulating the movement and dispersion of test bacteria of faecal contamination (E. coli) have been used in France on occasions as a tool in the positioning of sampling points, or even in the choice of sampling frequency, if the results of the simulations are concordant with the data observed by the REMI monitoring network. The models used combine a hydrodynamic model with one of bacterial decay and allow simulation of the distribution of bacterial concentrations and the impact of contaminations due to new discharges or changes in existing ones. A new standard operating procedure will be set up for all Ifremer laboratories for the use of a model appropriate for the study of the characteristics of the circulation of microbiological pollutants in the shellfish production areas.

In the UK, CEFAS has used a simple hydrodynamic and particle tracking model for the assessment of contamination impact in a small, number of harvesting areas. The model could be extended to other areas where appropriate depth and tidal information is available. Where more complex hydrodynamic models have been developed for specific sewage improvement schemes by other organizations, and the modeling has been available to CEFAS, the outputs have been used for assessment of impact on shellfisheries.

2.5.2 Shoreline surveys

A shoreline survey is a physical inspection of the land adjacent to a harvesting area in order to identify potential sources of contamination that may not have been revealed by the desk study. From the information available to the working group, it is not apparent that shoreline
surveys are undertaken in any Member State prior to the commencement of monitoring for the purposes of classification. Information on the location and nature of potentially contaminating sources therefore relies on the use of written records or databases, often provided by other authorities.

Shoreline surveys may be undertaken in some Member States when abnormally high results have been seen in a particular harvesting area.

2.5.3 Bacteriological surveys

A bacteriological survey is a preliminary monitoring programme, undertaken subsequent to the initial desk study that establishes a baseline for the bacteriological contamination of the area. This may be undertaken purely for the purpose of determining the siting of sentinel monitoring points for ongoing monitoring or may be used to justify an initial classification of the area, pending ongoing monitoring.

Monitoring of harvesting areas for the purposes of classification is implicit in the requirements of Directive 91/492. This initial monitoring is seen as separate to the ongoing monitoring of harvesting areas stipulated under Chapter VI of the Annex to the Directive. There is no stipulation as to the number of results needed prior to classification, nor to the period of time over which these should be obtained in order to allow for variations in bacterial concentrations with fluctuations in environmental factors such as season, meteorological conditions, tide, etc.

In England and Wales, a provisional classification is established on the basis of at least 10 results taken over at least a minimum 3-month period. Harvesting is not allowed prior to the establishment of a provisional classification. A full classification is only established after a full year of monthly monitoring. These requirements may be modified if there is historical and ongoing data from a different species at the same point, or the same species at nearby points, and parallel monitoring of the species/points shows that the levels of contamination are comparable.

In France, a classification is established on the basis of a sanitary study with at least 26 results, by sampling point, over a one or two year period. Prior to the establishment of this classification, harvesting may be allowed on a provisional basis in the case of natural offshore beds or remote areas, for example, depending on the results of ongoing monitoring.

In Italy, initial classification is achieved on the basis of 6 results over a 6 month period - maintenance monitoring is then undertaken every 3 months.

2.6 Approaches taken in non-EU countries

The US National Shellfish Sanitation Programme (NSSP) Model Ordinance gives detailed requirements for the conduct of sanitary surveys. Such approaches are also used in other countries that have export agreements with the US (e.g. Canada, Mexico, New Zealand).

There are three principal components to the NSSP sanitary survey: a desk-based collection of relevant data; a physical shoreline survey looking for potential sources of contamination, together with a baseline bacteriological monitoring programme.
Comprehensive sanitary surveys are undertaken for harvesting areas where there is no previous data, the existing data is out of date, or where there are known changes in the potentially contaminating sources.

A report is prepared which includes the following:

- Overview of shellfishery
- Fishery
  - Bivalve species
  - Aquaculture or wild stocks
  - Seasonality of harvest
  - Harvesting techniques
  - Any conservation controls
- Hydrography/hydrodynamics (this may involve dilution calculation, mathematical modelling, dye tracking)
- Location, size and treatment level of human sources of contamination
- Location and estimated volume/load of agricultural sources of contamination
- Significant wild animal/bird populations
- Maps, seasonality effects, for these factors
- Records of shoreline surveys
- Records of baseline bacteriological monitoring results
- Assessment of effect on contamination of shellfish

In practice, much of this information will be assembled from existing sources. It is also the case that the assessment of the contamination effects of particular sources on the bivalve molluscs will be subjective rather than objective. This will often be the case with data on wild animal/bird populations, or even agricultural sources of contamination, where a very large study would be needed to convert population values to estimates of bacterial concentrations in seawater or bivalve molluscs. A significant proportion of the content sanitary survey reports may therefore consist of information that does not practically affect the siting of sample points, the frequency of timing of sampling, or the interpretation of the monitoring programme results.

Annual reviews are undertaken for all harvesting areas to ensure that the environmental conditions have not changed and that the classifications are still valid. This process includes:

a) file review on the status of all bivalve mollusc growing areas;
b) performance records for all sewage treatment works and industrial discharges;
c) a status report on abatement of pollution from sources identified during past sanitary surveys;
d) evaluation of new pollution sources; and
e) bacteriological sampling at representative stations at a suitable frequency if deemed necessary from the results of items a)-d).

Once every three years a complete re-evaluation is undertaken, although there is an allowance for this to be undertaken less frequently for remote areas deemed to be of low risk of contamination. There is also the allowance to dispense with the need for regular water monitoring if there are no identified pollution sources in the vicinity of the shellfishery.
The NSSP also includes specific details for the evaluation of marinas and adjacent waters.

Harvesting areas may be classified as Prohibited (no harvesting allowed) with respect to microbiological factors if they are within or adjacent to marinas (but see above); if no current sanitary survey is in existence; the area is adjacent to a sewage treatment plant outfall or other point source outfall with public health significance; pollution sources may unpredictably contaminate the growing area; the area is contaminated with faecal waste so that the bivalves may be vectors for disease microorganisms.

2.7 Discussion

A number of Member States with bivalve mollusc harvesting areas undertake some form of assessment of contaminating sources prior to establishing the siting of monitoring points. Following the acquisition of a significant amount of data, a risk assessment of the area may be used to determine subsequent monitoring frequency. The approach to sanitary surveys will need to be formalized, at least for newly classified areas, after the introduction of the Hygiene 3 Regulation in January 2006.

There is considerable experience elsewhere in the world with regard to the application of sanitary surveys (e.g. USA, Canada, New Zealand). However, it will be useful to determine exactly which parts of these studies actively contribute to the development of sampling plans, etc, so that resource is not wasted in collecting large amounts of information that will not be used constructively.

The following paragraph from the Canadian Shellfish Sanitation Programme is pertinent to the practical application of sanitary surveys: “The assessment of sanitary quality of shellfish areas is becoming increasingly difficult. The large-scale urbanization of many of our coastal areas has resulted in the proliferation of non-point pollution sources which are difficult to identify and quantify. The relationship between indicator organisms and pathogens, particularly viruses, is becoming less clear and the sanitary significance of hinterland drainage has been questioned. Chemical contamination is of concern in many coastal areas. It is necessary, therefore, to apply a conservative approach to growing area classification, particularly where faecal contamination has been verified but the origin is unknown, or where chemical discharges may affect shellfish growing areas.”.

Changing environmental conditions also complicate the assessment of the significance of potentially contaminating sources and the achievement of classifications that fully reflect the potential risk from the harvested bivalve molluscs.
Table 2.2 Elements of sanitary surveys undertaken in Member States

<table>
<thead>
<tr>
<th>Member State</th>
<th>Mapping Hard copies</th>
<th>Mapping GIS</th>
<th>Discharges</th>
<th>Agricultural data</th>
<th>Other sources taken into account</th>
<th>Assessment of influences</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belgium</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>No information received</td>
</tr>
<tr>
<td>Denmark</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>No information received</td>
</tr>
<tr>
<td>France</td>
<td>Y</td>
<td>Y</td>
<td>Record of continuous and intermittent discharges - national RNDE database</td>
<td>Y</td>
<td>Food industries, camp sites</td>
<td>Mathematical 2D modelling (case-by-case basis)</td>
<td></td>
</tr>
<tr>
<td>Germany</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>No information received</td>
</tr>
<tr>
<td>Greece</td>
<td>Y</td>
<td>N</td>
<td>Record of continuous and intermittent discharges</td>
<td>N</td>
<td>Industrial wastes River inputs</td>
<td>Tide charts used to assess impact</td>
<td></td>
</tr>
<tr>
<td>Ireland</td>
<td>Y</td>
<td>Y</td>
<td>Record of discharges</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Member State</td>
<td>Mapping Hard copies</td>
<td>Mapping GIS</td>
<td>Discharges</td>
<td>Agricultural data</td>
<td>Other sources taken into account</td>
<td>Assessment of influences</td>
<td>Comment</td>
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</tr>
<tr>
<td>Italy</td>
<td>Y</td>
<td>Y Gauss-Boaga method</td>
<td>Record of continuous and intermittent discharges</td>
<td>Y</td>
<td>Industrial wastes Surface water overflows River inputs (volume and season) - Tides (type and amplitude) - precipitations (volume and frequency) - winds (according to the season and effect on pollution dispersion)</td>
<td>Pattern of sampling points varied according to contaminating influences</td>
<td></td>
</tr>
<tr>
<td>Netherlands</td>
<td>Y</td>
<td>N</td>
<td>Record of continuous and intermittent discharges</td>
<td>N</td>
<td>Only when necessary Tides Precipitations Winds Fisheries activities</td>
<td>Assessment of influences is based on expert judgement</td>
<td></td>
</tr>
<tr>
<td>Portugal</td>
<td>Y</td>
<td>N</td>
<td>Record of discharges</td>
<td>N</td>
<td>“Other runoffs”</td>
<td>Case-by-case basis</td>
<td></td>
</tr>
<tr>
<td>Spain</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Information on contaminating inputs is not taken into account when determining sample sites</td>
<td></td>
</tr>
<tr>
<td>Member State</td>
<td>Mapping Hard copies</td>
<td>Mapping GIS</td>
<td>Discharges</td>
<td>Agricultural data taken into account</td>
<td>Other sources</td>
<td>Assessment of influences</td>
<td>Comment</td>
</tr>
<tr>
<td>------------------------</td>
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<td>----------------------------------------------------------------------------</td>
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<td>--------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Sweden</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UK</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>England &amp; Wales</td>
<td>N</td>
<td>Y</td>
<td>National database available (position, volume, discharge type, treatment level)</td>
<td>Summary data available</td>
<td></td>
<td>Not on a systematic basis</td>
<td>Tide charts used to assess impact Sample sites selected to reflect the impact of contaminating sources</td>
</tr>
<tr>
<td>Scotland</td>
<td>N</td>
<td>Y</td>
<td>Information available from Scottish Environmental Protection Agency</td>
<td>Data available from Local Food Authority</td>
<td></td>
<td>Not on a systematic basis</td>
<td>Sample sites principally selected to monitor each aquaculture operation</td>
</tr>
<tr>
<td>UK Northern Ireland</td>
<td>Y</td>
<td>Y</td>
<td>Information available from Department of Environment</td>
<td></td>
<td></td>
<td>Not on a systematic basis</td>
<td>Sample sites selected to monitor each aquaculture operation</td>
</tr>
</tbody>
</table>
3. Sampling plans - bivalve mollusc species, spatial and temporal considerations
[How to decide what to sample, where, and how often]

3.1 Introduction

The results obtained in a microbiological monitoring programme will depend on the design and implementation of the programme and, in statutory programmes, this will have a direct effect on the compliance determined using the results – in terms of bivalve molluscs, this will affect the classification status of harvesting areas. The four principal factors shown to affect results are the species sampled, the location of sampling points (primarily in relation to sources of contamination), the frequency of sampling and the choice of data set (period of time, tolerance allowed). Considerations given here interact with those relating to sanitary surveys and effects of environmental factors given in Section 2.

3.2 Bivalve species

Lart & Hudson (1993) investigated the differences in E. coli concentrations in several species sampled in the same geographical location. They did detect significant differences between species but these varied depending on the season. The work included only a small number of sampling occasions and thus the results would have been complicated by the other factors affecting individual E. coli concentrations in bivalves. In the monitoring programme in England & Wales, a general tendency has been shown for the degree of contamination to be in the order (from highest to lowest):

1) mussels (Mytilus edulis); flat oysters (Ostrea edulis); Manila clams (Ruditapes philippinarum)
2) Pacific oysters (Crassostrea gigas)
3) Other clams, including razor clams (Ensis spp). Scallops (Pecten maximus).

The relative E. coli content of cockles (Cardium edule) appears to vary with location and may reflect the nature of the seabed substrate amongst other factors. Cockles appear to have a greater tendency to show intermittent high results that are not explained by known contamination factors.

The observed species differences probably reflect the interaction of a number of different factors including their biological activity (rate of uptake and depuration) and location in the water column/substrate (see Section 3.3 below). The biological activity will reflect innate differences in activity and will be affected by season, water temperature and salinity. Low salinities may result in some species stopping their filtration activity altogether and this may mean that they are not exposed to the full contamination effects of rainfall-associated contamination episodes.

The factors described above will affect the detection of contamination episodes in different species. For example, following a breakdown at a sewage works, mussels will normally show a quick increase, followed by decrease, in contamination whereas the event will affect E. coli levels in Pacific oysters more slowly and the levels in these will remain higher for longer after the contamination event is over.
There is little information as to whether the differences observed in *E. coli* contamination between bivalve species reflect differences in pathogen content. The French Veterinary Services found 0.9% *Salmonella* (77 positive results in 8377 samples) in live bivalve molluscs from dispatch centres (Raffin 1994). The *Salmonella* positive rate varies according to the bivalve species: oysters (0.2%), mussels (1.1%), others bivalves such as cockles or manila clams (1.7%). In salt marsh and estuaries conditions, 28 samples (4.6%) were found positive for *Salmonella* in 614 manila clams samples (Catherine 1995). Most of the positive *Salmonella* results (68% of the 28 samples) were detected in highly polluted areas (results > 4600 *E. coli*) and the average contamination level was above $10^4$ *E. coli*/100g of bivalve molluscs. Similar information is not available for viral contaminants. There is also no information as to species-specific rates of uptake and clearance of pathogens that may be seen following short-term contamination events such as a sewage treatment plant breakdown. Although differences in biological activity of individual bivalve mollusc species might be expected to affect indicators and pathogens similarly, the markedly different depuration rates for bacterial indicators and viral pathogens (Lees 2000) may affect any such relationship.

### 3.3 Spatial effects

Spatial effects include two dimensional considerations (geographical location) and also depth, including position in relation to the seabed material. Lart & Hudson (1993) studied *E. coli* concentrations at regular points in grids with overall dimensions varying from tens of metres to 1.5 kilometres. The grids were located in different harvesting areas in the UK. Significant differences in mean *E. coli* content were detected on scales as small as metres, but the effect varied between harvesting areas. In some, but not all cases, the significant differences could be related to physical factors such as distance from presumed contamination sources. Similar results were obtained in studies on French harvesting areas (Beliaeff 1992).

Belliaeff and Cochard (1995) undertook a detailed study of spatial variation across a French bouchots mussel farming area (approximate dimensions 6.5km x 0.9km) on two separate sampling occasions. They found significant variation in faecal coliforms concentrations across the area. They concluded that attempts to obtain an overall picture of spatial contamination across a bed may be prohibitive in terms of cost and suggested that a practical solution would be to restrict sampling to the points representing the highest level of faecal contamination.

Younger *et al.* (2003) noted that variations were often found in the extent of *E. coli* contamination seen in mussels along the length of cultivation ropes and this is assumed to be the result of contamination being confined to particular depths due to density gradients. Some effects may also result from the resuspension of contaminated sediment and differences in the extent of contamination may be seen in the same species grown on the seabed and on trestles in the same area.

### 3.4. Temporal effects

The effect of sample time on *E. coli* concentrations is largely influenced by a number of the factors discussed in Section 2.3, together with those discussed in 3.2 above. These factors combine to yield the possibility for *E. coli* concentrations to vary by up to approximately $10^4$ per 100g over a period of hours (Tattersall *et al.* 2003; Younger *et al.* 2003). The magnitude of the variation will depend on the level of contamination in the area, as well as the effects of
the environmental and biological factors. An average variation up to $10^2$ E. coli per 100g of bivalve molluscs was found in estuary condition between samples collected during low tides of springtide and low tides of neap tide (Catherine et al. 1995). The differences in time that may be found between peak E. coli concentrations in different species following a contamination event was noted above. It is therefore not possible to use individual E. coli results to predict the level of contamination over even a period as short as one day and this invalidates the use of small numbers of results to determine short-term changes in classification status.

### 3.5 Legislative requirements

From a geographical perspective, Article 5, 2. (a) of Directive 91/492 identifies that “The competent authority shall establish a list of production and relaying areas, with an indication of their location and boundaries, from which live bivalve molluscs may be taken in accordance with the requirements of this Directive…”. Chapter 1 of the Annex states that “The location and the boundaries of production areas must be fixed by the competent authority in such a way as to identify the areas from which live bivalve molluscs:” meet the classification requirements.

Chapter VI of the Annex to the Directive identifies that sampling plans for the periodic monitoring of live bivalve mollusc relaying and production areas must take into account likely variations in faecal contamination at each production and relaying area. This is presumed to include consideration of variations in both space and time. No further details are given as to how this should be determined. No consideration of species-specific effects within a single area is given in the Directive.

Annex 3.A of the Hygiene 3 Regulation states that the competent authority must “establish a sampling programme of bivalve molluscs in the production area which is based on the examination of established data, and with a number of samples, a geographical distribution of the sampling points and a sampling frequency which must ensure that the results of the analysis are as representative as possible for the area considered.”.

Annex B.2 of the Regulations additionally states that: “To implement paragraph 1(b), (c) and (d), sampling plans must be drawn up providing for such checks to take place at regular intervals, or on a case-by-case basis if harvesting periods are irregular. The geographical distribution of the sampling points and the sampling frequency must ensure that the results of the analysis are as representative as possible for the area considered.”. In addition, B.3 identifies that “Sampling plans to check the microbiological quality of live bivalve molluscs must take particular account of:
(a) the likely variation in faecal contamination, and
(b) the parameters referred to in paragraph 6 of Part A.”.

The first part of which is identical to the requirement in the current Directive while the second part cross-refers to the requirements for sanitary surveys. The new Regulations are therefore much more explicit with regard to the need to take into account both spatial and temporal variation. However, there is still the possibility for differences in interpretation with regard to siting of monitoring points and the frequency of sampling, together with the subsequent interpretation of the results.
3.6 Practices within EU Member States

In this Section, the following parts of the procedures for monitoring and classification of production areas (stable and permanent or variable) are summarised as they are found in the information forwarded from the NRLs of the Member States. The wide variety in approaches means that it is not possible to undertake a detailed critique and the information is presented in the form of tables.

There are some areas where there is general agreement in approach.

1. Most Member States map the location of their bivalve mollusc fisheries and associated monitoring points, either as hard copies or in Geographical Information Systems (GIS).
2. Most Member States identify fixed monitoring points within the fisheries/classified areas. An exception is Denmark, where samples are taken from wherever within a zone commercial fishing is currently taking place.
3. Most Member States take one sample from each monitoring point on each sampling occasion. An exception is the Netherlands, where 5 samples are taken at the same point/occasion.

In the Tables for this section, the content of sampling plans are summarised by:

1. **Molluscan shellfish species (aquaculture and wild fisheries) (Table 1)**
   a) Sentinel species used for classification and monitoring
   b) Group of species
   c) All species

2. **Spatial considerations including (Tables 2, 3, 4 and 5)**
   a) Definition or the legal decision of the size of a single production area or areas in total
   b) The size of permanent and variable A classified production areas and of B and C
   c) The size of production areas for intertidal fisheries and for relaying areas
   d) Number of sampling points and minimum sampling frequency and frequency and number of samples taken in A, B and C areas
   e) The depth at which samples are taken, the length from fixed sampling points that samples can be taken, where the samples are taken and how they are taken e.g. “bagged “or not.

3. **Temporal considerations including (Table 6)**
   a) Are samples taken during closed seasons
   b) Are the samples taken randomised
   c) Are the samples targeted at the most polluted and representative spot
   d) Sampling in variable areas.
It shall be noted that live echinoderms, live tunicates and live marine gastropods will be included in the summary tables wherever it has been possible to obtain information on these species.

3.7 Practices in other countries

In the US, and other countries following the system laid down in the NSSP, sampling is either undertaken on a “worst case” or a systematic random basis. For the former, samples have to be collected under conditions that have been identified as yielding worse results. In general, a minimum of 5 samples have to be collected a year from each sample point and a minimum of the 15 most recent samples are used to determine compliance. For systematic random sampling, collection dates and times are scheduled in advance (e.g. annually) in order to ensure random collection with respect to environmental conditions. In this case, a minimum of 6 random samples have to be collected each year from each sample point and a minimum of the 30 most recent samples are used to determine compliance. There are allowances for reductions in sampling frequency for sites deemed remote from pollution sources and for those that are commercially inactive. It could be viewed that the “worst case” approach yields the best public health protection while the systematic random sampling approach yields a better estimate of the average level of contamination. There are problems with both approaches. With the “worst case”, it can take the results of a large number of samples and detailed analysis to convincingly show the whole range of environmental conditions (rain, tide, wind, etc) under which the higher levels of contamination will occur. With systematic random sampling, sampling may be scheduled for dates and times when it is not possible to undertake fieldwork, e.g. due to bad weather or adverse tides.

3.8 Summary of the review given in Tables 3.1 to 3.8

It is clear from the review, Member States have markedly different approaches to the establishment and use of sampling plans and that a more harmonized approach would be a valuable asset in improving the management of the microbiological classification and monitoring of the production areas. From the information summarized in the tables it can be seen that more detailed information is needed in order to provide better comparison on the details of the different systems.

In general, Member States have not specified whether they use only one species of bivalve shellfish, a group of species or all the commercial species in a production area for the classification and for the monitoring. This needs to be clarified.

Only few member states have given any information on the size of the production areas. It is important to gather knowledge of the experience the member states have with the size of an area in contrast to the number of sampling points and to the number of samples to be taken, how they are taken, the depth they are taken at, the spreading over racks or ropes, if they are “bagged” samples, and the procedures for sampling via “bagged” samples. It is important to get established where a production area can be “large” and where it must be “small”. It is also important to get established where 1 sampling point is sufficient, where several sampling points must be established and where variable sampling points are the most sensible way of sampling. But how shall “large” and “small” production areas be defined?

Almost no information was given on relaying areas, and no specific information was given about the subtidal or intertidal fisheries with regard to the sampling of bivalve mollusc
species, spatial and temporal considerations. An intertidal fishery is “the collection of wild bivalve molluscs on the sea bottom, while the tide is out”.

Concerning the number of production areas versus the number of permanent sampling points the information gathered was poor and did not give any possible conclusions to the relation between the two. Most of the member states gave information on the frequency of sampling and this information is useful in the discussion of the minimum frequency for sampling in the A, B and C classified production areas. Further comparison of the current practices in Member States would be aided by the provision of more detailed information.

It would be valuable to consider further the practices given under the NSSP and to determine how one or the other of such approaches might be pursued within Europe.
Table 3.1 Overview of Molluscan shellfish species used as sentinel species as well as all species from wild fisheries and from aquaculture.

<table>
<thead>
<tr>
<th>Country</th>
<th>Sentinel species used for classification and monitoring</th>
<th>Group of species used for classification and monitoring</th>
<th>All species from aquaculture and wild fisheries</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belgium</td>
<td>European Oyster (<em>Ostrea edulis</em>) and Pacific Oyster (<em>Crassostrea gigas</em>)</td>
<td></td>
<td>European Oyster (<em>Ostrea edulis</em>) and Pacific Oyster (<em>Crassostrea gigas</em>)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Wild fishery of scallops and marine gastropods are caught outside of the production area.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>The areas where the scallops are caught are known, but they are not production areas.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>The areas where the marine gastropods are caught are not known; they are caught outside the production area.</td>
</tr>
</tbody>
</table>
| Denmark   | Blue mussels (*Mytilus edulis*) in most of the cases, but any other specie can be used, if it is the bivalve mollusc to be fished or harvested. 
| Finland¹  | No current harvesting areas | | |
### Sentinel species used for classification and monitoring

<table>
<thead>
<tr>
<th>Country</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>France</td>
<td><em>Buccinum undatum, Paracentrotus lividus, Cerastoderma edule, Ruditapes decussatus, Ruditapes philippinarum, Venerupis rhomboides, Donax trunculus, Venus verrucosa, Glycymeris glycymeris, Mercenaria mercenaria, Spisula ovalis</em></td>
</tr>
<tr>
<td></td>
<td>Pacific oyster (<em>Crassostrea gigas</em>), European oyster (<em>Ostrea edulis</em>), mussels (<em>Mytilus edulis, Mytilus galloprovincialis</em>)</td>
</tr>
<tr>
<td>Germany, Lower Saxony Federal State</td>
<td>Blue mussels <em>Mytilus edulis</em> and oysters (species?)</td>
</tr>
<tr>
<td>State Niedersachsen</td>
<td></td>
</tr>
<tr>
<td>Germany, Schleswig-Holstein</td>
<td>Blue mussels <em>Mytilus edulis</em> and oysters (species?)</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Greece</td>
<td>Mussels (<em>Mytilus galloprovincialis</em>), cockles, Smooth Venus (<em>Venus verrucosa</em>) and <em>Modiola barbata</em></td>
</tr>
</tbody>
</table>

### Group of species used for classification and monitoring

<table>
<thead>
<tr>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>gastropods, echinoderms and tunicates</td>
<td>bivalve molluscs that live in sediment</td>
<td>others filter-feeding bivalve molluscs</td>
</tr>
</tbody>
</table>

### All species from aquaculture and wild fisheries

<table>
<thead>
<tr>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Buccinum undatum, Paracentrotus lividus, Patella vulgaris, Littorina littorea, Haliotis tuberculata, Microcosmus sabatieri</em></td>
</tr>
<tr>
<td><em>Cerastoderma edule, Ruditapes decussatus, Ruditapes philippinarum, Venerupis rhomboides, Donax trunculus, Donax vittatus, Venus verrucosa, Glycymeris glycymeris, Mercenaria mercenaria, Spisula ovalis, Spisula solida,</em></td>
</tr>
<tr>
<td>Pacific oyster (<em>Crassostrea gigas</em>), European oyster (<em>Ostrea edulis</em>), mussels (<em>Mytilus edulis, Mytilus galloprovincialis</em>), scallops (<em>Pecten maximus, Aequipecten opercularis, Chlamys varia</em>)</td>
</tr>
<tr>
<td>Blue mussels <em>Mytilus edulis</em> and oysters (species?)</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Blue mussels <em>Mytilus edulis</em> and oysters (species?)</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Mussels (<em>Mytilus galloprovincialis</em>), cockles and Smooth Venus (<em>Venus verrucosa</em>), <em>Modiola barbata, Donax trunculus and oysters Ostrea edulis</em></td>
</tr>
<tr>
<td>Country</td>
</tr>
<tr>
<td>-----------</td>
</tr>
<tr>
<td>Ireland</td>
</tr>
<tr>
<td>Italy</td>
</tr>
<tr>
<td>Luxemburg</td>
</tr>
<tr>
<td>Netherlands</td>
</tr>
<tr>
<td>Portugal</td>
</tr>
<tr>
<td>-----------</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>
|           | Bivalves from the sandy, muddy bottoms of the coast: surf clams (*Spisula solida*), donax clams (*Donax* spp.), razor clams (*Ensis* spp.), smooth venus clams (*Callista chione*), scallops (*Pecten maximus*), striped venus (*Venus gallina*). | | Spain, general information
<pre><code>       | There are 9 autonomous coastal regions of which only 3 have given information. | | |
</code></pre>
<p>| Spain, Galicia | Rock mussel is mainly used, but also other species like clams, cockles, etc | | Rock mussel, clams, cockles, etc? |</p>
<table>
<thead>
<tr>
<th>Location</th>
<th>Sentinel species used for classification and monitoring</th>
<th>Group of species used for classification and monitoring</th>
<th>All species from aquaculture and wild fisheries</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spain, Andalucia</td>
<td><em>Mytilus edulis</em>, <em>Chamelea gallia</em>, <em>Callista chion</em>, <em>Donax trunculus</em>, <em>Ruditapes decussatus</em>, <em>Ruditapes philippinarum</em>, <em>Venerupis rhomboidea</em>, <em>Scrobicularia plana</em>, <em>Cerastoderma edule</em>, <em>Paracentrotus lividus</em>, <em>Mytilus galloprovincialis</em>, <em>Venus nux</em>, <em>Anemonia sulcata</em></td>
<td>Each Production area has a principal control specie, but due to variety in ecosystems a great number of species are analysed</td>
<td><em>Mytilus edulis</em>, <em>Chamelea gallia</em>, <em>Callista chion</em>, <em>Donax trunculus</em>, <em>Ruditapes decussatus</em>, <em>Ruditapes philippinarum</em>, <em>Venerupis rhomboidea</em>, <em>Scrobicularia plana</em>, <em>Cerastoderma edule</em>, <em>Paracentrotus lividus</em>, <em>Mytilus galloprovincialis</em>, <em>Venus nux</em>, <em>Anemonia sulcata</em></td>
</tr>
<tr>
<td>Spain, Cataluna</td>
<td>The species are not given, but the specie must representative for the aquaculture and for the fishery is used as indicator.</td>
<td></td>
<td>The species are not given</td>
</tr>
<tr>
<td>Sweden</td>
<td>Blue mussels (<em>Mytilus edulis</em>)</td>
<td></td>
<td>Blue mussels (<em>Mytilus edulis</em>) and oysters</td>
</tr>
</tbody>
</table>
Table 3.2 Overview of the size of classified production areas for Wild Fisheries and for Aquaculture (including Intertidal Fisheries and Relaying areas - unless information is available, in which case it is given in table 3.3)

<table>
<thead>
<tr>
<th>Country</th>
<th>Definition or the legal decision of the size of the single production areas</th>
<th>The size of an A classified production areas. Permanent classified areas</th>
<th>The size of a zone A area within a production area of B classification Variable classification of areas</th>
<th>The size of B classified production areas</th>
<th>The size of C classified production areas</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belgium</td>
<td>1 aquaculture establishment only in 1 production area. Wild fishery of scallops and marine gastropods are caught outside of production areas</td>
<td>1 aquaculture establishment only – with permanent to variable classification. 86 ha inland saline pond connected through a lock with the port of Oostende and a canal.</td>
<td>10 – 20 square nautical miles</td>
<td>If found, then 10 – 20 square nautical miles</td>
<td></td>
</tr>
<tr>
<td>Denmark</td>
<td>Precisely delineated areas with geographical co-ordinates and names, given on maps. Size: 10 – 20 square nautical miles</td>
<td>No permanent</td>
<td>3 x 3 square nautical miles</td>
<td>10 – 20 square nautical miles</td>
<td>If found, then 10 – 20 square nautical miles</td>
</tr>
<tr>
<td></td>
<td>Definition or the legal decision of the size of the single production areas</td>
<td>The size of an A classified production areas. Permanent classified areas</td>
<td>The size of a zone A area within a production area of B classification Variable classification of areas</td>
<td>The size of B classified production areas</td>
<td>The size of C classified production areas</td>
</tr>
<tr>
<td>----------------------</td>
<td>--------------------------------------------------------------------------------</td>
<td>------------------------------------------------------------------------</td>
<td>--------------------------------------------------------------------------------</td>
<td>------------------------------------------</td>
<td>------------------------------------------</td>
</tr>
<tr>
<td>France</td>
<td>Precisely delineated areas with geographical co-ordinates are given on maps. The intertidal production areas cover in total 19000 hectares of production (aquaculture) and 1583 km of bouchots mussels (natural and offshore beds excluded).</td>
<td>Wide range of sizes (from 10 ha to 1000 ha).</td>
<td>No such zones.</td>
<td>Wide range of sizes (from 10 ha to 1000 ha).</td>
<td>Wide range of sizes (from 10 ha to 1000 ha).</td>
</tr>
<tr>
<td>Germany\textsuperscript{v}</td>
<td>Precisely delineated areas with geographical co-ordinates and given on maps.</td>
<td>No distinction between A; B or C areas according to the FVO-inspection report from 2002, since the lower limit for <em>E. coli</em> is 10,000 CFU/100 gram of molluscs. No information on size.</td>
<td>No information on size.</td>
<td>No information on size.</td>
<td>No information on size.</td>
</tr>
<tr>
<td>Greece</td>
<td>Precisely delineated areas with geographical co-ordinates and given on maps</td>
<td>No information on size</td>
<td>No information</td>
<td>No information</td>
<td>No information</td>
</tr>
<tr>
<td></td>
<td>Definition or the legal decision of the size of the single production areas</td>
<td>The size of an A classified production areas. Permanent classified areas</td>
<td>The size of a zone A area within a production area of B classification Variable classification of areas</td>
<td>The size of B classified production areas</td>
<td>The size of C classified production areas</td>
</tr>
<tr>
<td>----------------------</td>
<td>------------------------------------------------------------------------------</td>
<td>---------------------------------------------------------------------</td>
<td>------------------------------------------------------------------------------------------------</td>
<td>--------------------------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>Ireland</td>
<td>Precisely delineated areas with geographical co-ordinates and names, given on maps. No information on size.</td>
<td>No information on size.</td>
<td>No information on size.</td>
<td>No information on size.</td>
<td>No information on size.</td>
</tr>
<tr>
<td>Italy</td>
<td>Harvesting areas given on maps with sampling points.</td>
<td>No information on size.</td>
<td>No information on size.</td>
<td>No information on size.</td>
<td>No information on size.</td>
</tr>
<tr>
<td></td>
<td>Samples are taken of bivalve molluscs and water by drawing transects of 1500 m length, perpendicular to the shoreline at intervals of up to 2000 m apart. Samples are to be taken from at least 5 points within each transect – the first on the shoreline and the others at 250 m, 500m, 1000m and 1500m.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Netherlands</td>
<td>Precisely delineated areas with geographical co-ordinates and names, given on maps, but no information on size.</td>
<td>Precisely delineated areas with geographical co-ordinates and names, given on maps, but no information on size.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
|              | Definition or the legal decision of the size of the single production areas | The size of an A classified production areas. Permanent classified areas | The size of a zone A area within a production area of B classification
Variable classification of areas | The size of B classified production areas | The size of C classified production areas |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Portugal</td>
<td>Production areas defined by name and by geographical co-ordinates. The areas are announced in a national official journal. Classification is given to a cluster of wild fisheries and aquaculture, and not to the production area. (2003) The extent of the classified clusters is defined in the 2003 classification listing.</td>
<td>Classification is given to a cluster of wild fisheries and aquaculture, and not to the production area. (2003)</td>
<td>Classification is given to a cluster of wild fisheries and aquaculture, and not to the production area. (2003)</td>
<td>The extent of the classified clusters is defined in the 2003 classification listing.</td>
<td>Classification is given to a cluster of wild fisheries and aquaculture, and not to the production area (2002)</td>
</tr>
<tr>
<td>Spain</td>
<td>No information from Galicia and Andalucia. Cataluna: 23 production areas given on charts.</td>
<td>No information from Galicia, Andalucia, and Cataluna.</td>
<td>No information from Galicia, Andalucia and Cataluna.</td>
<td>No information from Galicia, Andalucia and Cataluna.</td>
<td>No information from Galicia, Andalucia and Cataluna.</td>
</tr>
<tr>
<td>Sweden</td>
<td>No information</td>
<td>No information on size</td>
<td>No information</td>
<td>No information</td>
<td>No information</td>
</tr>
<tr>
<td>UK England &amp; Wales</td>
<td>Precisely delineated areas with geographical co-ordinates and names, given on maps. No information on size</td>
<td>Of differing sizes</td>
<td>Production areas with more than 1 monitoring point can get more than 1 classification. Of differing sizes</td>
<td>No information on size</td>
<td>No information on size</td>
</tr>
</tbody>
</table>

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<table>
<thead>
<tr>
<th></th>
<th>Definition or the legal decision of the size of the single production areas</th>
<th>The size of an A classified production areas. Permanent classified areas</th>
<th>The size of a zone A area within a production area of B classification <strong>Variable classification of areas</strong></th>
<th>The size of B classified production areas</th>
<th>The size of C classified production areas</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>UK Scotland</strong></td>
<td>Precisely delineated areas with geographical co-ordinates and code numbers/names – generally relate to individual aquaculture operations</td>
<td>Of differing sizes</td>
<td>Of differing sizes</td>
<td>Of differing sizes</td>
<td>Of differing sizes</td>
</tr>
<tr>
<td><strong>UK Northern Ireland</strong></td>
<td>Precisely delineated areas with geographical co-ordinates and names generally related to individual aquacultural operations, size in hectares recorded</td>
<td>Of differing sizes</td>
<td>Of differing sizes</td>
<td>Of differing sizes</td>
<td>Of differing sizes</td>
</tr>
</tbody>
</table>

1. No information available.
2. The document from Lower Saxony Federal State Office for Consumer Protection and Food Safety, LAVES, did not contain any information on monitoring of bivalve molluscs.
4. No information available.
5. Portugal technical procedures, code PT02, ed. 02, date: 10.03.03
6. All information on from the 2 “Länder”, Niedersachsen and Schleswig-Holstein, is given under Germany.
Table 3.3  Overview of size of production areas for the Intertidal Fisheries and for Relaying-areas (within zone A areas), if specified

<table>
<thead>
<tr>
<th>Country</th>
<th>Size of production areas for the Intertidal Fisheries - if specified</th>
<th>Size of Relaying-areas within zone A areas - if specified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belgium</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Denmark</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>France</td>
<td>Wide range of sizes.</td>
<td>No classified relaying areas.</td>
</tr>
<tr>
<td>Germany</td>
<td>No information available</td>
<td>No information available</td>
</tr>
<tr>
<td>Greece</td>
<td>No information</td>
<td>None</td>
</tr>
<tr>
<td>Ireland</td>
<td></td>
<td>None</td>
</tr>
<tr>
<td>Italy</td>
<td>In Italy, maximum tidal excursion value is 1 mt</td>
<td>3 Relaying areas are classified – but no detailed information on the size.</td>
</tr>
<tr>
<td>Netherlands</td>
<td>No information</td>
<td>“Re-watering plots” (storage areas) are designated. They are always of A classification. Mussels lay there from 7 days to 3 months. No size of the areas is given.</td>
</tr>
<tr>
<td>Portugal</td>
<td>No information</td>
<td>No information</td>
</tr>
<tr>
<td>Spain</td>
<td>No information from Galicia, Andalucia and Cataluna.</td>
<td>No information from Galicia, Andalucia and Cataluna.</td>
</tr>
<tr>
<td>Sweden</td>
<td>No information</td>
<td>No information</td>
</tr>
<tr>
<td>United Kingdom</td>
<td>No information</td>
<td>No information</td>
</tr>
</tbody>
</table>

An intertidal fishery is “the collection of wild bivalve molluscs on the sea bottom, while the tide is out”.

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Table 3.4 Overview of total number of sampling points and the Frequency and number of samples taken in A, B, C and D production areas

<table>
<thead>
<tr>
<th>Country</th>
<th>Total number of permanent sampling points in production areas and in aquaculture</th>
<th>Minimum Frequency and number of samples taken in production areas in general</th>
<th>Frequency and number of samples taken in A</th>
<th>Frequency and number of samples taken in B</th>
<th>Frequency and number of samples taken in C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belgium</td>
<td>1 sampling point located up to 1 km from the harvesting place.</td>
<td>Every second week samples of oysters are taken.</td>
<td>Every second week</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 water samples per week for water quality (bathing water) from May to September.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denmark</td>
<td>No permanent sampling points in the wild fisheries.</td>
<td>Samples are not taken in inactive production areas</td>
<td>Monthly: 3 samples form 3 positions.</td>
<td>Ultimo 2004: weekly sampling by all fishermen and aquaculture establishments during active weeks</td>
<td>Ultimo 2004: weekly sampling by all fishermen and aquaculture establishments during active weeks</td>
</tr>
<tr>
<td></td>
<td>For aquaculture there are fixed sampling points at each aquaculture establishment</td>
<td></td>
<td>Monthly: 3 samples form 3 positions.</td>
<td>Monthly: every second month or every 3 months depending of microbiological stability of the area</td>
<td>Monthly: every second month or every 3 months depending of microbiological stability of the area</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Monthly: every second month or every 3 months depending of microbiological stability of the area</td>
<td>Monthly: every second month or every 3 months depending of microbiological stability of the area</td>
<td>Monthly: every second month or every 3 months depending of microbiological stability of the area</td>
</tr>
<tr>
<td>France</td>
<td>380 sampling points: most zones have 1 point but 66 have several points (from 2 to 6)</td>
<td>Every 2 weeks (intermittent fishing on natural beds), monthly or every second month. Generally, every 2 weeks over a 1 year period for a classification study.</td>
<td>Monthly or every 3 months depending of microbiological stability of the area</td>
<td>Monthly, every second month or every 3 month depending of microbiological stability of the area</td>
<td>Monthly or every 3 months depending of microbiological stability of the area</td>
</tr>
<tr>
<td>Germany</td>
<td>No information available</td>
<td>No information available</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Country</td>
<td>Total number of permanent sampling points in production areas and in aquaculture</td>
<td>Minimum Frequency and number of samples taken in production areas in general</td>
<td>Frequency and number of samples taken in A</td>
<td>Frequency and number of samples taken in B</td>
<td>Frequency and number of samples taken in C</td>
</tr>
<tr>
<td>-----------</td>
<td>----------------------------------------------------------------------------------------------------------------------------------------</td>
<td>-------------------------------------------------------------------------------------------------------------------------------------</td>
<td>------------------------------------------</td>
<td>------------------------------------------</td>
<td>------------------------------------------</td>
</tr>
<tr>
<td>Greece</td>
<td>Permanent sampling points every 2 km. Alternate sampling points can be used.</td>
<td>For classification 6 samples of bivalve molluscs and 6 samples of sea water, minimum, are examined every 15 to 30 days, for a period of 3 to 6 months, depending on the results. Monitoring programme: microbial check of water every 3 month, microbial check of bivalve molluscs every month.</td>
<td>Monthly.</td>
<td>Monthly.</td>
<td>Monthly.</td>
</tr>
<tr>
<td>Ireland</td>
<td>Sampling points given on maps with harvesting areas and all other essential information on each single area.</td>
<td>Minimum monthly. For classification 12 samples within 3 months.</td>
<td>Monthly</td>
<td>Monthly</td>
<td>Monthly</td>
</tr>
<tr>
<td>Italy</td>
<td>Sampling points given on maps with harvesting areas</td>
<td>Weekly / biweekly to 3 month (at least) but varies form region to region</td>
<td>Weekly / biweekly to 3 month (at least) but varies form region to region</td>
<td>Weekly / biweekly to 3 month (at least) but varies form region to region</td>
<td>Weekly / biweekly to 3 month (at least) but varies form region to region</td>
</tr>
<tr>
<td>Total number of permanent sampling points in production areas and in aquaculture</td>
<td>Minimum Frequency and number of samples taken in production areas in general</td>
<td>Frequency and number of samples taken in A</td>
<td>Frequency and number of samples taken in B</td>
<td>Frequency and number of samples taken in C</td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>Netherlands 13 of which is the “re-watering plots”</td>
<td>Every second week, when production areas are active. The re-watering plots are generally sampled weekly.</td>
<td>Every second week, when production areas are active.</td>
<td>Not applicable</td>
<td>Not applicable</td>
<td></td>
</tr>
</tbody>
</table>

WS04/013
| Portugal | 48 | Monthly, one for each production area. | Monthly. If 1 sample exceeds limits then Weekly. If 3 consecutive samples exceeds limits then declassified. Continued weekly sampling until 3 consecutive samples comply with limits - then reclassified. | Monthly. If 1 sample exceeds limits then Weekly. If 3 consecutive samples exceeds limits then declassified. Continued weekly sampling until 3 consecutive samples comply with limits - then reclassified. | Monthly. If 1 sample exceeds limits then Weekly. If 3 consecutive samples exceeds limits then closure/suspension. Continued weekly sampling until 3 consecutive samples comply with limits - then reclassified. |

Sample size:
30 units of *Mytilus* spp.
10 to 15 units of oysters, smooth venus clams, scallops and hardshell clams.
30 to 50 units of other species.
<table>
<thead>
<tr>
<th>Total number of permanent sampling points in production areas and in aquaculture</th>
<th>Minimum Frequency and number of samples taken in production areas in general</th>
<th>Frequency and number of samples taken in A</th>
<th>Frequency and number of samples taken in B</th>
<th>Frequency and number of samples taken in C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spain</td>
<td>Galicia: 87 fixed points for rock mussels, 18 fixed points for other bivalves and 90 other points of not continuous control. Andalucia: No exact information, maybe 45 to ?? Cataluna: No exact information. No permanent sampling points in the wild fisheries.</td>
<td>Cataluna: Monthly for all sampling points in May to August. Every second month the rest of the Year. Samples are not taken in inactive production areas.</td>
<td>Galicia: Monthly Andalucia: Monthly. Cataluna: Monthly for all sampling points in May to August. Every second month the rest of the Year. Samples are not taken in inactive production areas.</td>
<td>Galicia: Every second month. Andalucia: Every second month. Cataluna: No C areas</td>
</tr>
<tr>
<td>Sweden</td>
<td>No information</td>
<td>Monthly or every third month</td>
<td>Monthly</td>
<td>Every third month</td>
</tr>
</tbody>
</table>

WS04/013
<table>
<thead>
<tr>
<th>Total number of permanent sampling points in production areas and in aquaculture</th>
<th>Minimum Frequency and number of samples taken in production areas in general</th>
<th>Frequency and number of samples taken in A</th>
<th>Frequency and number of samples taken in B</th>
<th>Frequency and number of samples taken in C</th>
</tr>
</thead>
</table>
| UK England & Wales | 414 - sampling points given on maps. | Monthly  
10 samples, taken at each monitoring point during 3-4 months are necessary for a new classification. 
More samples may be required for an A classification. 
If offshore usually 6 sets of samples are taken for new classification 
Full classification requires regular monthly results for 1 year. | Monthly | Monthly | Monthly |
| UK Scotland | Samples taken from set grid reference point from within the production area boundary | Initial classification based on 6 samples (may all be taken on the same day for a fast-track classification) 
Full classification requires 6 more samples taken in separate months of the remaining year. | | | |
<table>
<thead>
<tr>
<th>Total number of permanent sampling points in production areas and in aquaculture</th>
<th>Minimum Frequency and number of samples taken in production areas in general</th>
<th>Frequency and number of samples taken in A</th>
<th>Frequency and number of samples taken in B</th>
<th>Frequency and number of samples taken in C</th>
</tr>
</thead>
<tbody>
<tr>
<td>UK Northern Ireland</td>
<td>Samples taken from within classification area boundaries</td>
<td>Initial provisional classification based on one sample per week for each species for 12 weeks. Full classification requires regular monthly sample results for one year.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Table 3.5 Overview of the depths and areas, at which the samples are taken and how the samples are taken.

<table>
<thead>
<tr>
<th>Aquaculture</th>
<th>Wild Fisheries</th>
<th>Intertidal production areas</th>
<th>Relaying areas</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 level of depth</td>
<td>From the sea bottom or from “Bagged” mussels at 1 level of depth.</td>
<td>From the sea bottom or from “Bagged” mussels.</td>
<td>From the sea bottom or from “Bagged” mussels at 1 level of depth.</td>
</tr>
<tr>
<td>2 levels of depth</td>
<td>Distance from nominal sampling point</td>
<td>Distance from nominal sampling point</td>
<td>Distance from nominal sampling point</td>
</tr>
<tr>
<td>3 levels of depth</td>
<td>“Bagged” mussels at 1 level of dept</td>
<td>“Bagged” mussels.</td>
<td>“Bagged” mussels at 1 level of dept.</td>
</tr>
<tr>
<td>“Bagged” mussels at 1 level of dept</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distance from nominal sampling point</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| Belgium | No information available | | |
| Denmark | 3 levels of depth | The sea bottom, where fishery is active. | The sea bottom |
| France | Depends on the type of aquaculture, from the bottom or from the ropes at a depth level as near as possible to the theoretical co-ordinates of the sampling point (indications are given in the sampling form for each point if necessary) | From the bottom as near as possible to the theoretical co-ordinates of the sampling point (indications are given in the sampling form for each point if necessary) | From the bottom, the trestles, the bouchots as near as possible to the theoretical co-ordinates of the sampling point as necessary (indications are given in the sampling form for each point if necessary) |
| | | | No relaying areas |
| Germany | No information | No information | No information |

**WS04/013**
<table>
<thead>
<tr>
<th>Country</th>
<th>Sampling Method</th>
<th>Sampling Radius</th>
<th>No information</th>
<th>No information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Greece</td>
<td>For cockles and smooth Venus: Samples taken in the centre of the aquaculture unit, at 1 level of depth near the sea bottom. For mussels: Samples taken in the centre of the aquaculture unit, at 3 levels of depth at top, middle and near the sea bottom.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ireland</td>
<td>Within 50 m from the sampling point.</td>
<td>Within 50 m from the sampling point.</td>
<td>No information</td>
<td></td>
</tr>
<tr>
<td>Italy</td>
<td>The sea bottom and “Bagged” mussels are used in certain areas.</td>
<td>The sea bottom and “Bagged” mussels are used in certain areas.</td>
<td>The sea bottom and “Bagged” mussels are used in certain areas.</td>
<td>The sea bottom and “Bagged” mussels are used in certain areas.</td>
</tr>
<tr>
<td>Netherlands</td>
<td>No information</td>
<td>No information</td>
<td>No information</td>
<td></td>
</tr>
<tr>
<td>Portugal</td>
<td>30 Meters in diameter from sampling point.</td>
<td>200 Meters in diameter from sampling point.</td>
<td>30 Meters in diameter from sampling point.</td>
<td>30 – 200 Meters in diameter from sampling point.</td>
</tr>
<tr>
<td></td>
<td>Spain</td>
<td>Andalucia</td>
<td>Cataluna</td>
<td>Sweden</td>
</tr>
<tr>
<td>------------</td>
<td>---------------------------------</td>
<td>-----------------------------------------------</td>
<td>-----------------------------------------------</td>
<td>--------</td>
</tr>
<tr>
<td>Galicia</td>
<td>3 levels of depth, at 1, 5 and 10 meters, and 15 animals are taken at each level.</td>
<td>No information – but sampling depth is noted on samples.</td>
<td>The sea bottom, where fishery is active.</td>
<td></td>
</tr>
<tr>
<td>Andalucia</td>
<td>No information – but sampling depth is noted on samples.</td>
<td>No information – but sampling depth is noted on samples.</td>
<td>No information – but sampling depth is noted on samples.</td>
<td></td>
</tr>
<tr>
<td>Cataluna</td>
<td>For rafts 3 places, beginning, middle and end of rafts. For long-line 1 or 2 samples depending on length of structure.</td>
<td>No information – but sampling depth is noted on samples.</td>
<td>No information – but sampling depth is noted on samples.</td>
<td></td>
</tr>
</tbody>
</table>

| Sweden     | Mainly near the top of the rope | No information                              | No information                              | No information | No information |
| United Kingdom | Depends on the type of aquaculture operation – a study will be undertaken on roped mussels to determine the most contaminated depth | Normally taken using the usual commercial practice for the fishery | Normally taken using the usual commercial practice for the fishery | Normally taken using the usual commercial practice for the fishery | No information |
Table 3.6 Overview of temporal considerations - when and how are samples taken

<table>
<thead>
<tr>
<th></th>
<th>Are samples taken during closed seasons</th>
<th>Are the samples taken randomised</th>
<th>Are the samples targeted at the most polluted and representative spot</th>
<th>Sampling in variable areas.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belgium</td>
<td>No</td>
<td>No</td>
<td>Permanent</td>
<td>No</td>
</tr>
<tr>
<td>Denmark</td>
<td>No</td>
<td>Yes</td>
<td>They are taken in the area, where the actual fishing is to go on</td>
<td>No</td>
</tr>
<tr>
<td>France</td>
<td>Yes</td>
<td>Yes, as much as possible</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>No sampling in prohibited (D) area</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Germany</td>
<td>No information available</td>
<td>No information available</td>
<td>No information available</td>
<td>No</td>
</tr>
<tr>
<td>Greece</td>
<td>NO</td>
<td>NO</td>
<td>No information available</td>
<td></td>
</tr>
<tr>
<td>Ireland</td>
<td>?</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Italy</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Netherlands</td>
<td>No</td>
<td>Yes</td>
<td>NO</td>
<td>NO</td>
</tr>
<tr>
<td>Portugal</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes, when possible</td>
<td>?</td>
</tr>
<tr>
<td>Sweden</td>
<td>No information</td>
<td>No</td>
<td>No information</td>
<td>No</td>
</tr>
<tr>
<td>United Kingdom</td>
<td>Reduced frequency may be agreed upon in inactive areas.</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>In prohibited areas, the frequency may be reduced or suspended.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
4. Sampling and sample transport for microbiological analysis

4.1 Introduction

Bivalve molluscs that are to be microbiologically examined for the purpose of the official control monitoring of harvesting areas are usually taken from those areas rather than being sampled from harvesters, or at purification or dispatch centres, in order to ensure that they have been taken from the designated monitoring point(s) (as dictated by the sampling plan) and under the appropriate controlled conditions. Depending on the type of shellfishery, it may be necessary to be sampled by boat. Many of the harvesting areas are situated remote from both samplers’ offices and from laboratories. The sample time is usually dependent on tidal cycles and accessibility of the area, except in the Mediterranean Sea, which results in difficult timing from sampling through transportation of the samples. All of these factors mean that sampling and sample transport need to be carefully planned and sufficient resources made available to ensure that the data obtained from the sampling programme is accurate.

The results of the monitoring programme will depend largely on the sources of contamination in an area together with spatial, temporal and environmental effects. The method of analysis may also markedly affect results. These factors are dealt with in other Sections but some can be influenced, or even controlled, by the approach taken to sampling and need to be addressed within any sampling protocol. The method of sampling and the treatment of the sample during and after sampling are also very important. These factors include avoidance of contamination, sample bags/containers, transportation method, temperature control and maximum delay before analysis. This section will therefore focus on the main factors to be defined with regard to sampling and sample transport in connection with microbiological monitoring of harvesting areas. The section does not focus on identification of sampling site or frequency as this is covered in the previous section on sampling plans.

There is therefore a need to standardize sampling and sample transport procedures in order to ensure that samples that are analysed are representative of the bivalve molluscs at the sample point. Sampling officers need to be provided with a protocol containing details as to how samples should be taken, cleaned of sediment, packed and transported. Where samples are taken with the help of the industry, e.g. if an official boat is not available, it is preferable for this to be done under the supervision of a sampling officer. If this is not possible, sampling protocols and relevant training need to be provided and audits undertaken to ensure compliance with the protocol.

The following have been identified as necessary components of a sampling protocol:

a. The location and type of sample
   This is required in order to reduce variability introduced by spatial variation and the differences in extent of contamination between species (Lart & Hudson 1993).

b. The means of sampling
   Additional contamination may be introduced during some commercial harvesting practices such as dredging and, where possible, it is best to sample using the means normally used for commercial harvesting in the area. Where this is not possible, or where an indicator species is being used, samples may be taken by other means (e.g. hand-picked) or bagged bivalve molluscs may be kept at the monitoring point for the purpose
of sampling. With the latter, the effect of location in the water column should be considered. It is also important to avoid the bivalves ingesting sediment disturbed by the sampler during the collection process.

c. Number of individual animals forming the sample (by species)
Individual animals sampled at the same location and time will vary markedly in their faecal coliforms and \textit{E. coli} content (Lart & Hudson 1993). It is important to specify the collection of sufficient animals to minimize this effect and also, particularly for the smaller species, to allow sufficient flesh and intravalvular fluid to perform the microbiological test. The number of animals should be sufficient to allow for some mortality in transit and still provide sufficient animals to meet the other requirements.

d. Cleansing of the exterior shells of samples
There is the potential for the bivalves to be contaminated after sampling and this needs to be avoided. Bivalves covered with dirt, sediment, algae and other organisms may become contaminated inside the storage bag. There is a need to use clean equipment fit for the purpose and to undertake proper cleaning of the sample. It is also necessary to avoid the samples becoming immersed in fluid as they may then take up or excrete contaminants including the bacteria of interest.

e. Sampling record (perhaps on sample submission form)
It is necessary to ensure that samples are labelled accurately and that a proper record of sampling details are kept in order to ensure traceability of the sample.

f. Sample containers and outer packaging to be used.
The use of suitable containers is necessary to avoid contamination of individual samples and to avoid cross-contamination between different samples.

g. Temperature control during transportation
h. Acceptable time lag between sampling and analysis
Faecal coliforms and \textit{E. coli} do not tend to multiply in seawater or bivalve mollusc samples stored at \(10^\circ\text{C}\) or less (Cook & Ruple 1989; Lart & Hudson 1993). Prolonged storage at low temperatures may result in reductions in counts and it is recommend that samples are stored (whether in transport or otherwise) at a temperature of less than 8\(^\circ\text{C}\) and that the maximum time lag between sampling and analysis is 24 hours. Properly packed cool boxes containing ice packs (not in direct contact with the samples) should achieve a temperature less than 8\(^\circ\text{C}\) within 4 hours and maintain this for at least 24 hours. Samples should not be frozen as this will result in a marked decrease in faecal coliforms/\textit{E. coli} concentrations. Dead shellfish should never be used for analysis.

4.2. Sampling and sample transport practices in Member States

Detailed information on practices was only received from France, Greece, Ireland, Netherlands, Portugal and the UK. Less detailed information was received from some other Member States. The following items were addressed in the information that was received.

Place of sampling: This is usually required to be at a monitoring point defined in the sampling plan or identified to be in the centre of an aquaculture or other harvesting area. A tolerance around the monitoring point may be specified. In Denmark, samples (minimum of 3) are taken from wherever within a harvesting area commercial activity is being undertaken.
Time of sampling: If defined in the protocol, this is usually either required to be on as random a basis as possible (UK: England and Wales) or at a specific state of tide (France: low water of spring tide in Channel and Atlantic ocean).

Sampling method: Where mentioned in protocols, collection of bivalves by the normal commercial harvesting practice is usually required or preferred. The placing of bagged bivalves at the monitoring point for sampling purposes is allowed in some systems.

Number of animals per sample: Most protocols specify the (minimum) number of animals that should constitute a sample. For oysters, for example, the number specified is in the region of 10-18 while for mussels the number is 18-35 (most being around 25-30). Some protocols specify shellfish volumes in order to ensure sufficient sample.

Cleaning of samples: A number of protocols identify that the bivalves should be washed/rinsed in clean seawater or potable water and then drained before being placed in the sample bags/containers.

Sample bags/containers: The most commonly specified are plastic bags. A small number of protocols use net bags. (See Table 4.1)

Cool boxes: Most, but not all, protocols stipulate the use of appropriate insulated boxes containing cool packs. (See Table 4.1)

Maximum transit time/temperature: The stipulated maximum period ranges from 24 (most common) to 48 hours. In Galicia, the samples invariably reach the laboratory within 4 hours. (see Table 4.1)

Labeling of samples: Where specified, this usually requires the use of waterproof labels which may contain the following information: sample point details; date and time of collection; name of sampler. The samples may also be labeled with a sample number, in combination with a registration form including all relevant data.

Sample submission forms: Where standard submission forms were provided, these contained at least the information recorded on the sample label plus one or more of the following: collection method; species; tidal state; meteorological information; observations on any abnormal conditions at time of sampling (e.g. sewage debris, storm discharges operating; large numbers of animals/birds in the vicinity). Space may also be given for recording time of receipt at the laboratory, temperature on receipt and time of commencement of analysis. An example of a sample form can be found in Annex 3.

Available information on sample transport conditions is given in Table 4.1 and example protocols are given in Annex 1 for France and Annex 2 for the UK.
Table 4.1. Methodology of transport, packaging material, transportation material and method, Controls and Rejection requirements of microbiological molluscan shellfish samples within the EU.

<table>
<thead>
<tr>
<th>Member State</th>
<th>Time sampling-analysis</th>
<th>Packaging material</th>
<th>Material</th>
<th>Method</th>
<th>Controls</th>
<th>Rejection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belgium</td>
<td>&lt;24 hours</td>
<td>Plastic Bag</td>
<td>Portable refrigerator</td>
<td>Courier</td>
<td>Temperature</td>
<td>Unknown</td>
</tr>
<tr>
<td>Denmark</td>
<td>&lt;24 hours</td>
<td>Plastic Bag</td>
<td>Cool Box</td>
<td>Courier</td>
<td>Delay</td>
<td>&gt;24 hours</td>
</tr>
<tr>
<td>France</td>
<td>&lt;24 hours</td>
<td>Plastic Bag</td>
<td>Cool Box</td>
<td>Courier</td>
<td>Temperature</td>
<td>&gt;15°C</td>
</tr>
<tr>
<td>Germany</td>
<td>&lt;24 hours</td>
<td>Plastic Bag</td>
<td>Cool Box</td>
<td>Courier</td>
<td>Unknown</td>
<td>None</td>
</tr>
<tr>
<td>Greece</td>
<td>&lt;24 hours</td>
<td>Plastic Bag</td>
<td>Cool Box</td>
<td>Courier</td>
<td>Viability</td>
<td>&gt; 24 hours</td>
</tr>
<tr>
<td>Ireland</td>
<td>&lt;24 hours</td>
<td>Plastic Bag</td>
<td>Cool Box</td>
<td>Courier</td>
<td>None</td>
<td>&gt;24 hours</td>
</tr>
<tr>
<td>Italy</td>
<td>&lt;24 hours</td>
<td>Plastic Bag</td>
<td>Insulator Bags</td>
<td>Own Transport</td>
<td>Temperature Vitality checks</td>
<td>Dead molluscs</td>
</tr>
<tr>
<td>Netherlands</td>
<td>Approx. 24 hours</td>
<td>Plastic Bag</td>
<td>Cool Box</td>
<td>Courier</td>
<td>Occasional</td>
<td>Insufficiently cooled Time &gt;36 hours</td>
</tr>
<tr>
<td>Portugal</td>
<td>Approx. 24 hours</td>
<td>Plastic/net bag</td>
<td>Cool Box</td>
<td>Regular transport</td>
<td>Viability check</td>
<td>Molluscs must be alive</td>
</tr>
<tr>
<td>Spain (Cataluna, Andalucia)</td>
<td>&lt;24 hours</td>
<td>Unknown</td>
<td>Cool Box</td>
<td>Courier Mail Own transport</td>
<td>None</td>
<td>Unknown</td>
</tr>
<tr>
<td>Spain (Galicia)</td>
<td>&lt;4 hours</td>
<td>Unknown</td>
<td>Portable refrigerator</td>
<td>Temperature</td>
<td>Temperature &gt; 18°C</td>
<td></td>
</tr>
<tr>
<td>Sweden</td>
<td>Unknown</td>
<td>Unknown</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UK England &amp; Wales</td>
<td>&lt;24 hours</td>
<td>Double plastic bag</td>
<td>Cool Box</td>
<td>Courier Mail Own transport</td>
<td>Delay</td>
<td>&gt;24 hours</td>
</tr>
<tr>
<td>UK Scotland</td>
<td>Usually &lt;24h – up to 48h for remote islands</td>
<td>Polythene bag</td>
<td>Cool Box</td>
<td>Courier Mail Own transport</td>
<td>Delay</td>
<td>&gt;48 hours</td>
</tr>
<tr>
<td>UK Northern Ireland</td>
<td>&lt;24 hours</td>
<td>Plastic bag</td>
<td>Cool Box</td>
<td>Own transport</td>
<td>Delay</td>
<td>&gt;24 hours</td>
</tr>
</tbody>
</table>
Annex 1: Collection of shellfish samples, transport and storage conditions in France

Collection of shellfish samples

For purposes of maximal comparability between separate results from the same area, it is essential to ensure the constancy of a large number of factors from one sample to another: the time and level of the tide, the time of day, the precise location where the sample was collected, and the size of the area in which the sample was taken.

Thus, sampling is performed systematically in the same conditions throughout the year, during periods of the day in which maximum activity by producers is likely, e.g. at low tide of springtide in the case of the English Channel and the Atlantic Ocean.

A representative sample is obtained by random collection of shellfish of similar size at various locations of the sampling unit (in plastic containers for oysters on tray, in the sand, on stakes, etc.), as determined at the sampling point concerned. The shellfish collected should be at least of commercial size for smaller species (mussels, cockles, etc.) and of small size for larger species (e.g. oysters). Juvenile shellfish, commonly referred to as spats, as well as those of less than commercial size, should not be collected.

The number of shellfish collected should be slightly greater than the amount required for the preparation of the mix to be analysed (70 to 100 g of flesh and intravalvular liquid), so as to eliminate those which are damaged, i.e. in general for the main species:

- oysters: 8-10 individuals
- mussels: 25-30 individuals
- cockles: 25-30 individuals
- clams: 25-30 individuals

“When sampling, the shellfish should be cleaned, if necessary, from excessive sediment and washed with clean seawater or potable water before being placed in an closed and labeled plastic bag” (French standard Afnor NF V 08-600 October 2000). From the taking in situ to their placement in the transport vehicle, the shellfish are protected against high temperatures.

Each sample is identified with a label including the following information:

- date :
- time :
- number and name of the point :
- shellfish species :
- sampler code :
- programme code :
  - regular survey :
  - or event :
- observations :

Equipment for sample collection

- strong, leakproof, single-use plastic bags
- sample labels
- tool for collecting non filter-feeding shellfish
- pocket knives and oyster knives
- a pair of rubber gloves
- an isothermal cooler
- cold packs
- thermometers
Transport and storage of shellfish samples

"The samples should be transported in an isothermal box at a temperature maintained by cold packs between +2 °C and +15 °C. The trip to the laboratory should be made as promptly as possible (NF V 08-600 October 2000).

During the transport of samples in a vehicle, the isothermal box should be kept away from sunlight. The temperature should be measured upon arrival at the laboratory (COFRAC Accreditation French Committee, programme No. 59/04 of September 1999).

"Upon arrival at the laboratory, the samples should be stored at a temperature ≤ 6 °C. Start the analysis as soon as possible; in any case the interval between collection and the beginning of the analysis should not exceed 24 hours” (NF V 08-600 October 2000).
Annex 2: Recommendations of the UK NRL for the collection and transport of bivalve molluscs for the classification of bivalve mollusc harvesting areas under Directive 91/492/EEC

May 2004

1. Introduction

The Food Safety (Fishery Products and Live Shellfish) (Hygiene) Regulations 1998 require the designation of bivalve mollusc production areas. This necessitates the sampling and testing of bivalve molluscs in order to determine the extent of sewage (or other faecal) contamination. This sampling only needs to be undertaken for areas from which bivalves are being taken to be placed on the market. Wild, but not farmed, scallops are excluded from these requirements.

3. Time of sampling

Sampling should be undertaken, where practical, on as random a basis as possible with respect to likely influencing environmental factors e.g. tidal state, rainfall, wind etc so as to avoid introducing any bias to the results.

4. Sampling method

Wherever possible, species should be sampled by the method normally used for commercial harvesting as this can influence the degree of contamination.

5. Size of individual animals

There is at least anecdotal evidence to suggest that immature/juvenile shellfish may give E. coli results that are unrepresentative of mature stock that will be harvested for commercial sale/human consumption. For this reason, all efforts should be made to ensure that only mature shellfish are sampled. The local Sea Fisheries Committee species-specific size limits may be used as a guide to what constitutes an adult shellfish.

6. Sample composition

The following sample sizes (in terms of number of individuals by species) are recommended for submission to the laboratory:

- Oysters and clams 12 - 18
- Mussels 18 - 35
- Cockles 35 - 55
- Razor clams (and other larger species) 8 - 10

This includes a small allowance over the number of individuals that need to be tested by the laboratory in case animals become moribund during transit.
6. Preparation of samples

Any mud and sediment adhering to the shellfish should be removed. This is best achieved by rinsing/scrubbing with clean seawater or fresh water of potable quality. If these are unavailable the seawater from the immediate area of sampling may be used instead. Do not totally re-immerse the shellfish in water as this may cause them to open. Allow to drain before placing in a food grade plastic bag. The container/bag should be labelled with the sender’s reference number and any other relevant information (e.g. species).

7. Sample transport

A cool box containing freezer packs should be used to maintain the temperature as near to 4°C as possible. Samples should be delivered to the laboratory as soon as practicable but the maximum time between collection and commencement of the test should not exceed 24 hours. Samples should not be frozen and freezer packs should not come into direct contact with the samples.

The cool boxes used for such transport should be validated using appropriate temperature probes, to ensure that the recommended temperature is achieved and maintained for the appropriate period. The number and arrangement of freezer packs, and the sample packing procedure, shown to be effective in the validation procedure should be followed during routine use. Where validation data already exists for a specific type of cool box, there is no need to undertake a local revalidation.

8. Submission form

Sample point identification number and name, map co-ordinates, time and date of collection, species sampled, method of collection (hand-picked, dredged, etc) and seawater temperature should be recorded on the submission form. Any other information deemed relevant (e.g. unusual events, adverse weather conditions etc) should also be recorded.

9. Additional advice

Additional advice on sampling can be obtained from Mr Andy Younger at the CEFAS Weymouth Laboratory: telephone: 01305 206695; e-mail: a.d.younger@cefas.co.uk
Annex 3. Example of a sample registration form.

<table>
<thead>
<tr>
<th>PROGRAMME CODE/DESCRIPTION</th>
<th>OPTIONAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample Code</td>
<td></td>
</tr>
<tr>
<td>Date</td>
<td></td>
</tr>
<tr>
<td>Time</td>
<td></td>
</tr>
<tr>
<td>Number of location</td>
<td></td>
</tr>
<tr>
<td>Location name</td>
<td></td>
</tr>
<tr>
<td>Shellfish species</td>
<td></td>
</tr>
<tr>
<td>Tidal Phase</td>
<td></td>
</tr>
<tr>
<td>Sampler code</td>
<td></td>
</tr>
<tr>
<td>Observations</td>
<td>Animals/Birds/overflows/vessels/tourism/etc.</td>
</tr>
<tr>
<td>Sample method</td>
<td>Dredge / hand pick /</td>
</tr>
<tr>
<td>Water temperature</td>
<td>Optional</td>
</tr>
<tr>
<td>Wind (direction and speed)</td>
<td></td>
</tr>
<tr>
<td>Rain</td>
<td>Yes / No</td>
</tr>
<tr>
<td>Arrival date</td>
<td>Optional</td>
</tr>
<tr>
<td>Arrival Time</td>
<td>Optional</td>
</tr>
<tr>
<td>Rejected</td>
<td>Yes / No</td>
</tr>
</tbody>
</table>
5. Comparability of testing methods

Microbiological monitoring of live bivalve mollusc harvesting areas is currently based upon the enumeration of *E. coli* or faecal coliforms as an indicator of faecal contamination under Directive 91/492 (Shellfish Hygiene Directive). Regulation (EC) No 854/2004 of the European Parliament and of the Council laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption (Hygiene 3) is due to be enacted in January 2006 and specifies the use of *E. coli* but not faecal coliforms.

5.1. Test methods for *E. coli* and/or faecal coliforms specified in EU legislation

Both the Shellfish Hygiene Directive and Hygiene 3 Regulation specify the use of a five-tube, three-dilution MPN method for the microbiological testing undertaken in connection with the microbiological monitoring of harvesting areas. In Directive 91/492, there is also the possibility of using “any other bacteriological procedure shown to be of equivalent accuracy” for use in testing LBMs intended for immediate human consumption (i.e. for operators’ own-checks, or competent authority audit checks, at purification and dispatch centres). One major difference between the current and proposed legislation is that classification will be based solely upon the results of *E. coli* monitoring of bivalve molluscs from the harvesting areas. There is no reference to the use of faecal coliforms as a monitoring tool.

Version 11 of the draft Commission Regulation on microbiological criteria for foodstuffs (European Communities 2004b) includes a reference analytical method for the enumeration of *E. coli* in live bivalve molluscs and live echinoderms, tunicates and gastropods. This is ISO 16649-3 (ISO 2004) and is based on the method described in Donovan *et al.*, 1998. The use of alternative analytical methods is deemed acceptable “when the methods are validated against the reference method set in Annex and certified by a third party in accordance with the protocol set in EN/ISO standard 16140 or other internationally accepted similar protocols. If the food business operator wishes to use analytical methods other than those validated and certified as described above, the methods shall at least be well documented and scientifically validated.”. No comparable reference procedure is given for testing undertaken for the microbiological monitoring of harvesting areas, either in Directive 91/492 or the Hygiene 3 Regulation. Also, no allowance is made for the use of any method that does not conform to the five-tube, three-dilution MPN procedure.

Also relevant to the matter of test methodology, method validation and proficiency testing are resolutions 4 and 5 of the 2004 meeting of the EU reference laboratory network for monitoring bacteriological and viral contamination of bivalve molluscs. These are given below:

4. Further to the opportunity to comment on the current proposals for revision of Community food legislation the NRLs recommended to the Commission and Member States that Hygiene 3 (European Communities 2004a) should include a reference method for *E. coli* testing in bivalve molluscs to mirror that found in the Microbiological Criteria for Foodstuffs (SANCO/4198/2001 rev 11; European Communities 2004b).
5. Further to the above the NRLs recommended that the Donovan method (Donovan et al., 1998, *Communicable Disease and Public Health*, 1, 188-196) should be the stipulated reference method for *E. coli* analysis in bivalve molluscs.

5.2 Overview of Methods of Enumeration

The most commonly used techniques used for the enumeration of *E. coli* and faecal coliforms in bivalve molluscs are the Most Probable Number Technique (MPN), direct counting on agar plates and rapid, automated or semi automated techniques. The latter include conductance/impedance techniques such as the Malthus and BacTrac. Within each of these enumeration techniques there are variations both in terms of the media that may be used and the precise format of the method used under the technique itself.

The limit of detection and the limit of quantification for any chosen test are important points for consideration i.e. the chosen test must be able to yield a numerical result lower than the regulatory limit. In the case of bivalve molluscs this is <230 *E. coli*/100g for class A. The limit of quantification will be determined by a combination of both the theoretical properties of the test method and the sensitivity of the media chosen.

5.2.1 MPN

MPN is a well-established technique for the enumeration of organisms in samples. Only viable, cultivatable organisms are enumerated by MPN determination. One or more dilutions of the original sample are divided into sub-samples, which are then added to media in tubes and incubated. A positive tube indicates the presence of one or more of the relevant microorganisms in the tube. The final MPN estimate is based upon the number of positive tubes obtained at the different dilution levels. The possible combination of positive tubes is limited and thus the final result is a categorical value. Two common variations of the MPN technique are the 3 tube by 3 dilution and the 5 tube by 3 dilution methods and these inherently possess different levels of variability. The 5x3 MPN method recommended by the Community Reference Laboratory has a nominal lower limit of 20 *E. coli*/100g calculated from the total quantity of bivalve mollusc meat and liquor actually inoculated into all tubes. Formal assessment of the performance characteristics of the method has been undertaken and it is intended to submit the results for publication.

5.2.1.1 Microbial Considerations

Whichever format is used, the first stage of an MPN test may be a resuscitation step with a relatively non-inhibitory growth medium. In the case of coliform media, this may include an acidity indicator to check for the fermentation of lactose e.g. minerals modified glutamate broth (MMGB). Such a resuscitation step allows time for damaged/stressed organisms to recover sufficiently to be able to grow and thus be ‘detected’. This is particularly relevant for environmental samples (such as bivalve molluscs) where *E.coli* is subject to various stresses e.g. osmotic. This first stage is often followed by subculture to another medium for confirmation that the target organism is actually present.

Some MPN methods use a more selective confirmatory style first stage medium such as brilliant green bile broth (BGBB). Differences in choice of first stage medium can potential lead to discrepancies in the results obtained if stressed organisms are present but not detected
due, perhaps, to a lack of opportunity to recover. Also the choice of second stage confirmatory media (post MPN tube) may also influence the final result, depending on the sensitivity and specificity of the media chosen.

High densities of non-coliform bacteria and the inhibitory nature of some lactose-based MPN media may have an adverse influence on routine coliform monitoring procedures. Many species in the general bacterial population have been shown to inhibit the detection of \textit{E. coli} (Waksman, 1941; Hutchison \textit{et al.}, 1943). Seidler \textit{et al.} (1981) showed that the recovery of total coliforms from water by MPN decreased as the concentration of heterotrophic bacteria (HPC) increased, with the greatest reduction occurring when the HPC densities exceeded 250 cfu/mL. Le Chevallier and McFeters (1985) hypothesized that competition for limiting organic carbon was responsible for the interference with total coliform recovery by HPC bacteria. The recovery of coliforms from gas-negative but turbid MPN tubes has demonstrated the presence of inhibitory compounds in the MPN media. When lauryl trehalose broth was the primary medium, subculturing turbid gas-negative tubes for confirmation testing increased the numbers of positive tubes in an MPN analysis by as much as 28\% (McFeters \textit{et al.} 1983). Comparative studies using brilliant green lactose bile (BGLB) broth and m-Endo LES agar as confirmatory media also demonstrated that BGLB broth can inhibit the growth of some coliforms.

Abbiss \textit{et al.} (1981) concluded that BGBB was more inhibitory than lauryl sulphate trehalose broth (LTB) or MMGB when enumerating coliforms in cheese, cooked meat and pate. The authors explained that because of the undefined nature of the bile salts (being present in the ox bile used in BGBB) variable results are possible as the inhibitory properties of bile salts vary considerably. Aruajo \textit{et al.} (1995) reported that faecal coliform counts in bivalve mollusc samples were markedly underestimated using the official Spanish MPN method, based on a first stage in BGBB, compared to a method using lauryl sulphate trehalose broth.

\textbf{5.2.1.2 Uncertainty of the Donovan MPN method}

The uncertainty of analysis in microbiology is usually given as twice the within laboratory standard deviation of the method. If this is to be quoted with the analytical results, this should be determined in the reporting laboratory and updated periodically. The following information is given as a general guide to the variability of the Donovan method, as determined in the Community Reference Laboratory.

The theoretical standard deviation of the 5-tube 3-dilution MPN test, as identified by the Health Protection Agency, UK, is 0.26 on a log$_{10}$ scale. This leads to a theoretical uncertainty of ± 0.52.

In a report of an EU Working Group on Measurement Uncertainties with Respect to Microbiological Analyses, the uncertainty determined in the CRL, determined by ANOVA of the results of a structured research study, was given as +/-0.6 log (Anon. 2003b). The analysis was undertaken using the MPNs taken from tables as exact values: i.e. no allowance was been made for a most probable range associated with the recorded MPN tube combinations. At low levels (<100/100g) near the nominal limit of detection of the test (20/100g), the uncertainty was found to rise to about +/-0.8 log.

For comparison, other estimates given in the report (the basis for the estimates were not explicitly defined) ranged from +/- 0.3-0.4 logs for aerobic plate counts to between +/- 0.4
and 1 log for many plate counts for indicators and pathogens. The estimates for the *E. coli* MPN for LBMs, obtained as defined above, was therefore within the range given for many other microbiological methods.

### 5.2.2 Plate Count

The spread plate and the pour plate methods utilise solid media and then relate the number of colonies formed to the viable count of the sample. The average number of colonies per plate is multiplied by the reciprocal of the dilution factor and by the reciprocal of the sample volume to determine the viable count. The nominal limit of detection of plate count methods is usually much greater than 230 *E. coli* per 100g unless either multiple standard size, or extra large, Petri dishes are used.

Direct plating on solid media does not tend to allow good recovery of stressed cells. Some media, such as MacConkey agar may also lead to a reduction in estimated numbers of coliforms (or faecal coliforms/*E. coli*) compared to other media as explained in section 5.1. MacConkey media contain bile salts, which may inhibit coliforms and *E. coli*, as well as the non-target bacteria when cells have been exposed to sublethal stress, as in the marine environment.

Some NRLs have expressed interest in the use of plate count methods using chromogenic media. Such methods may have the advantage of a reduced test period and reduced staff costs compared with the MPN procedure while avoiding the needs for the high equipment costs associated with the impedance procedure. However, there is a need to ensure proper validation of such procedures, particularly ensuring that the limits of detection and quantification are adequate and that the method allows adequate recovery of stressed cells. With regard to the latter, it is important that validation is undertaken using naturally, and not artificially, contaminated samples.

### 5.2.3 Conductance/Impedance methods

Impedance methods are used in a number of applications and work either by measuring electrical conductivity changes in liquid culture medium, due to charged end-products produced by growing bacteria (direct impedimetry), or by conductivity changes in a reaction solution brought about by the adsorption of gases from the inoculated bacterial culture (indirect impedimetry). Positive detection is based on the electrical changes exceeding a predetermined threshold within a defined time interval and enumeration is based on the time taken to exceed this threshold (detection time, DT). As the DT is inversely proportional to the log number of bacteria in the sample, bacterial counts can be predicted from the DT. A calibration is initially required to establish an experimental mathematical relationship between DT and log bacterial concentration. Two general standards based on impedimetry for microbiological examinations have been published, one by DIN in Germany (DIN 10115; Anon 1999) and the other by AFNOR in France (NF V08-105; Anon 200b).

The Malthus and Bactrac methods, as used for *E. coli*, are direct impedimetric techniques. Malthus coliform broth with incubation at 44°C has been used for the enumeration of *E. coli* by IFREMER, France, in their shellfish monitoring programme. The impedance method for the enumeration of *E. coli* in live bivalve molluscs has been standardized by AFNOR (NF V08-106; Anon 2002).
Dupont and Catherine (2003) found the uncertainty of the predicted \(E.\ coli\) concentrations for Malthus and BacTrac 4100, based on single detection times, as ± 0.63 and ± 0.58 respectively. The use of duplicate observations reduced this to ±0.45 and ±0.41 respectively, which is lower than that of single observations using the 5x3 tube MPN method. It should be noted that alternative methods calibrated against a reference method (such as an MPN test) will contain an element of variability due to that reference method.

### 5.2.4 Comparison of Impedance v MPN

Odumeru and Marrow (Personal communication) conducted a study to determine the sensitivity and specificity of the impedance-based microbiological method as a screening method for the detection of \(E.\ coli\) in foods within 24 hours of testing (part of the Enhanced Food Quality and Safety Research Program 1993-1998). A Malthus microbiological analyser system (Malthus System V, Malthus Instruments Ltd., Bury, United Kingdom), a modified Malthus Coliform Broth Medium (MCBM), and an incubation temperature of 44°C were used. The specificity of the impedance method was determined by testing \(E.\ coli\)-negative food samples spiked with \(Klebsiella\ pneumoniae\), \(Enterobacter\ cloacae\) and \(Pseudomonas\ aeruginosa\). The test results were compared with those obtained by the Most Probable Number (MPN) method. Milk, milk products, raw and ready-to-eat meats, and vegetables were tested for the presence of \(E.\ coli\). The sensitivity of the impedance method and the MPN method for detection of foods containing 101 CFU/g (i.e. 10100/100g) was 100% and 84.4% respectively. Both methods had a specificity of 100% for food samples spiked with 101 CFU/g \(E.\ coli\). The specificity of the impedance and the MPN methods for the detection of \(E.\ coli\) in naturally contaminated milk and meat samples was 100% and 95.7% respectively. \(E.\ coli\) was detected in foods by the impedance method within 4-24 h of testing at a detection limit of 1 CFU/ml. Bivalve molluscs were not tested in this study.

Jawad \textit{et al.} (1998) analysed both naturally contaminated food samples and spiked samples. The sensitivity of the MPN method was lower (84.4%) than that of the Malthus method (100%). Both methods had a specificity of 100% for food samples spiked with 10 CFU/g \(E.\ coli\). However the specificity of the MPN method was lower (95.7%) than the Malthus method (100%) in the case of the naturally contaminated samples. Bivalve molluscs were not included in this study.

Ogden \textit{et al.} (1998) indicated that a ‘small but significant number of samples tested positive on the Malthus instrument but were recorded negative on the MPN and Chromocult tests’. They went on to state that ‘subsequent analysis positively identified \(E.\ coli\) from these Malthus assays’. These \(E.\ coli\) were found not to be atypical, being lactose positive, and therefore should have been detected by the MPN and Chromocult methods.

Another consideration is the presence of other microorganisms could possibly produce false positive results. Dupont \textit{et al.} (1994) tested the selectivity of the conductance response with Malthus Coliform Broth incubated at 44 °C, which proved satisfactory, using 86 bacterial strains representing 45 different species. \(E.\ coli\) strains all exhibited an early conductance signal, whereas only one of five \(Klebsiella\ pneumoniae\) strains expressed a later conductance signal with an atypical conductance curve pattern. All the other strains showed no conductance response. In this study, interference of \(Kl.\ pneumoniae\) in mixed cultures with \(E.\ coli\) was deemed possible in instances where the concentration of \(Klebsiella\) was 1000 times more than that of \(E.\ coli\). For bivalve molluscs, the specificity of the impedance method was estimated at 99.2% from analyses performed on oysters, mussels, cockles and clams collected.
over a 2-year period in growing areas and natural beds (Dupont et al. 1996). In the few cases where false positive conductance responses occurred, impediometric estimations of bacterial concentration from DT were always less than 230/100 g.

Madden and Gilmour (1995) compared the MPN with the Rapid Automated Bacterial Impedance Technique (RABIT) for coliforms in milk. The false positive rate for the latter was 0.2%.

5.2.4.1 Calibration/Statistical Considerations

Dupont and Catherine (2003) undertook a validation exercise of both the Malthus and BacTrac 4000 and 4100 impedance systems using the French standard MPN Method (AFNOR NF V 08-600; Anon. 2000). Four different media were tested in the impedance systems. The BacTrac system was evaluated due to practical problems that were encountered in the use of the Malthus (lack of updating, maintenance and cost of impedance cells).

It was shown that there was a good linear response between log10 MPN E. coli and the detection time in the two systems. The relationships differed between the Malthus and BacTrac and also between the different media. With some of the equipment/media combinations, different relationships were seen with different bivalve species, which would have significant consequences with regard to calibration and testing. In such cases, different calibration lines must be used for testing. This can easily be accommodated in the systems.

5.2.4.2 Time and Cost Considerations

The recommended MPN method gives an E. coli estimation after 48 hours. The period of time is made up of 24-hour incubation period for the initial detection/resuscitation step and 24 hour for the confirmation phase. Dupont et al. (2004) concluded that the impedance method reduces analysis-handling time considerably and is much easier to use than the MPN method. Moreover, results can be obtained within 5-10 h, allowing rapid intervention to ensure public health protection in case of bivalve mollusc contamination. They suggest that the impedance measurement is a possible alternative to the MPN method for rapid quantitative estimation of E. coli in live bivalve shellfish.

The UK NRL conducted a ‘Consideration of MPN and Impedance procedures for the enumeration of Escherichia coli in bivalve molluscs’. They concluded that one of the biggest problems with the use of impedance technology in the UK was the high initial cost of the instrumentation – seventeen laboratories currently undertake classification testing in the UK. Consumables were estimated to cost in the order of £5 per test but savings in staff time were expected. Consideration of such an approach could not be considered viable unless bivalve mollusc testing was centralised and this could raise issues relating to sample collection and transport arrangements and time. There would also be validation/maintenance requirements if this impedance technology were to be considered for routine monitoring use. Some of these problems would be overcome if the instrumentation was also used for other testing purposes within the laboratories.

According to French NRL, the calibration/validation requirements represents the main cost of the impediometric system. Savings in staff time are very high with BacTrac 4300 system (20 ml disposable cells) compared to MPN technique in routine use (NF V 08-600), respectively 40 min. instead of 150 min per shellfish sample. Consumables (including two replicate per
sample) and maintenance costs were estimated at € 6.6 (€ 8.9 for MPN). The use of Bactrac 4300 system for LBMs was considered to be viable, compared to MPN technique, with less than 250 *E. coli* analyses per year per laboratory, including the equipment investment costs.

5.3 Comparision of Testing Methods Currently used by EU Member States with Active Harvesting Areas

The test methodologies used in the microbiological monitoring of harvesting areas are shown in Table 5.1. It is evident that a number of different testing methodologies are currently being employed in the determination of faecal contamination and ultimately the classification of harvesting areas for live bivalve molluscs (LBM). Both *E. coli* and faecal coliforms are currently used as monitoring tools within the EU, although the use of *E. coli* predominates.
### Table 5.1: Comparison of testing methods employed

<table>
<thead>
<tr>
<th>Member State</th>
<th>E.coli/Faecal coliforms</th>
<th>Method</th>
<th>Method format</th>
<th>Primary medium</th>
<th>Confirmation</th>
<th>FVO Reported method/year</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belgium</td>
<td></td>
<td>NF V08 600</td>
<td>5-tube 3-dilution MPN</td>
<td>Lauryl Sulphate Broth (LSB) 37°C/48h</td>
<td>EC broth 44°C/24h Peptone water 44°C/24h (then Kovacs)</td>
<td>F coliforms 5x3 MPN 2001</td>
<td>NF V08 600</td>
</tr>
<tr>
<td>Denmark</td>
<td>F coliforms MPN (presumptive E.coli)</td>
<td>Nordic Committee on Food Analysis Method no 96</td>
<td>5-tube 3-dilution MPN</td>
<td>Lauryl sulphate broth 37°C 2days</td>
<td>Thermotolerant coliforms: EC broth 44.5°C 1 day FC: tryptone water 44.5°C 1 day then Kovacs</td>
<td>F coliforms MPN 2001</td>
<td>NMKL No. 96 2nd ed. 1994</td>
</tr>
<tr>
<td>France</td>
<td>E. coli</td>
<td>NF V 08-600 MPN</td>
<td>5-tube 3-dilution MPN</td>
<td>Lauryl Sulphate Broth (LSB) 37°C/48h</td>
<td>EC broth 44°C/24h Peptone water 44°C/24h (then Kovacs)</td>
<td>AFNOR method NF V 08-600 October 2000</td>
<td></td>
</tr>
<tr>
<td>France</td>
<td>E. coli</td>
<td>NF V 08-106 Impedance</td>
<td></td>
<td>Impedance method using Malthus analyser</td>
<td></td>
<td>Validated impedance technique and MPN method 2001</td>
<td>Calibrated against NF V 08-600</td>
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<tr>
<td>Member State</td>
<td>E.coli/Faecal coliforms</td>
<td>Method</td>
<td>Method format</td>
<td>Primary medium</td>
<td>Confirmation</td>
<td>FVO Reported method/year</td>
<td>Comment</td>
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<tr>
<td>Greece</td>
<td>Faecal coliforms &amp; E. coli</td>
<td>ISO 7251</td>
<td>5-tube 3-dilution MPN</td>
<td>Lauryl sulphate broth 37°C 24-48h</td>
<td>EC broth 45°C 24-48h Indole 45°C 48h (then Kovacs)</td>
<td>Faecal coliforms 5x3 MPN 2001</td>
<td>ISO 7251:1993</td>
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<tr>
<td>Ireland</td>
<td>E. coli</td>
<td>Donovan</td>
<td>5-tube 3-dilution MPN</td>
<td>MMGB</td>
<td>TBGA</td>
<td>Donovan 2001</td>
<td>Copy of Test Method</td>
</tr>
<tr>
<td>Italy</td>
<td>F coliforms &amp; E. coli both criteria to be fulfilled</td>
<td>5x3 MPN</td>
<td>5-tube 3-dilution MPN</td>
<td>A1 medium 37°C 3h 44°C 24h</td>
<td>Tryptone water 44°C/24h (then Kovacs)</td>
<td>F coliforms &amp; E. coli both criteria to be fulfilled 2001</td>
<td>Studies are ongoing to replace this with the Donovan method</td>
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<tr>
<td>Netherlands</td>
<td>F coliforms</td>
<td>MacConkey plate count</td>
<td>Surface spread on agar plate</td>
<td>MacConkey Agar 37°C/2h 44°C/20-24h</td>
<td>BGBB 37°C/2h 44°C/20-24h</td>
<td>Mac Conkey plate count 2001</td>
<td>Old Dutch copy of Test Method</td>
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<td>Portugal</td>
<td>F coliforms</td>
<td>5x3 MPN</td>
<td>5-tube 3-dilution MPN</td>
<td>BGBB 37°C 1h/44°C 48h</td>
<td>None</td>
<td>Both methods included</td>
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<td>&quot;</td>
<td>E. coli</td>
<td>Donovan method</td>
<td>5-tube 3-dilution MPN</td>
<td>MMGB</td>
<td>TBX</td>
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<td>Member State</td>
<td>E.coli/Faecal coliforms</td>
<td>Method</td>
<td>Method format</td>
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<td>Confirmation</td>
<td>FVO Reported method/year</td>
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<tr>
<td>Spain</td>
<td>E.coli</td>
<td>Anon. 1991 5x3 MPN</td>
<td>5-tube 3-dilution MPN</td>
<td>BGBB 37°C/24-48h</td>
<td>Acid and gas (BGBB) &amp; Indole 44°C</td>
<td>JACUMAR established a national ‘Protocol of analysis of Faecal coliforms and E.coli’ 2001</td>
<td>Studies are ongoing to replace this with the Donovan method</td>
</tr>
<tr>
<td>Sweden</td>
<td>Faecal coliforms (presumptive E.coli)</td>
<td>MPN. Revised NMKL method</td>
<td>5-tube 3-dilution MPN</td>
<td>Lauryl sulphate tryptose broth 37°C 2days</td>
<td>Thermotolerant coliforms: EC broth 44.5°C 1 day FC: tryptone water 44.5°C 1 day then Kovacs</td>
<td>NMKL No. 96 2nd ed. 1994</td>
<td></td>
</tr>
<tr>
<td>UK</td>
<td>E. coli</td>
<td>Donovan</td>
<td>5-tube 3-dilution MPN</td>
<td>MMGB</td>
<td>TBGA</td>
<td>Donovan 2001</td>
<td>Copy of Test Method</td>
</tr>
</tbody>
</table>
5.4 Recommended Testing Procedure

The Donovan MNP method (Donovan et al. 1988) is the recommended procedure for the testing of bivalve molluscs within the EU. This method was agreed at a meeting of National Reference Laboratories for monitoring bacteriological and viral contamination of bivalve molluscs in 2002. This method used to enumerate \textit{E.coli} in bivalve molluscs is a two-stage; five tube by three dilution MPN method. The first stage of the method is a resuscitation requiring inoculation of minerals modified glutamate broth (MMGB) with a series of diluted bivalve mollusc homogenates and incubation at 37±1°C for 24±2 hours. The presence of \textit{E.coli} is subsequently confirmed by subculturing acid producing tubes onto agar containing 5-bromo-4-chloro-3-indoly-β-D glucuronide and detecting growth on the tryptone bile glucuronide agar (TBGA).

5.5 Validation of Alternative Methods Other Than Recommended CRL MPN Method

Current legislation requires that monitoring of harvesting areas for \textit{E. coli} or Faecal coliforms levels use a “five-tube, three-dilution MPN test”. There is no definition of the medium to be used or the temperature/time for incubation, all of which will affect the sensitivity and specificity of the procedure. As noted above, the method recommended by the reference laboratory network is the Donovan 5-tube 3-dilution MPN test using MMGB as the primary medium, with confirmation by plating on an agar to detect β-glucuronidase production. This method is due to be published as ISO TS 16649-3 in the autumn. The use of tests other than a 5-tube 3-dilution MPN does not currently conform to the requirements of the legislation. However, it is evident from Table 1 that other methods are being used and, if the use of these is to continue, they should be validated against the reference method. It would also be good practice for other MPN methods to be validated against the reference method.

The Hygiene 3 Regulation contains the same testing requirements with regard to harvesting area monitoring as the current Directive. The reference laboratory network considered this at their 2004 meeting and passed the following resolutions:

4. Further to the opportunity to comment on the current proposals for revision of Community food legislation the NRLs recommended to the Commission and Member States that Hygiene 3 (Official Controls Regulation – 2004/C48E/03) should include a reference method for \textit{E.coli} testing in shellfish to mirror that found in the Microbiological Criteria for Foodstuffs (SANCO/4198/2001 rev 9).

5. Further to the above the NRLs recommended that the Donovan method (Donovan et al., 1998, Communicable Disease and Public Health, 1, 188-196) should be the stipulated reference method for \textit{E.coli} analysis in bivalve molluscs.

6. Further to the above the NRLs recommended that the Hygiene 3 (Official Controls Regulations) should also include the possibility to use an alternative method for \textit{E.coli} analysis that was validated according to accepted scientific principles.

With respect to the validation of alternative methods, there are a number of published procedures. These include:
1. International Standard ISO 16140, Microbiology of food and animal feeding stuffs—
Protocol for the validation of alternative methods
This establishes the general principles of, and technical protocols for, the validation of
alternative methods in the field of microbiological analysis of food for:
- the validation of alternative methods which can be used in particular in the
framework of the official control;
- the international acceptance of the results obtained by alternative methods.
The document contains separate protocols for the validation of quantitative and
qualitative methods. With respect to the former, it contains requirements for testing
linearity, detection and quantification limits, sensitivity and specificity. It includes
recommendations for the number and type of samples to be included in comparative
testing and how such testing should be performed.

2. NordVal Validation protocol, Protocol for the validation of alternative microbial
methods.
This NordVal protocol describes the technical procedures for validation of alternative
methods for microbial analysis of food, water, animal faeces, feed, and food
environment samples in the Nordic countries. It includes similar requirements to the
ISO protocol. However, the ISO document is more detailed.

The work involved in conducting a full validation to ISO 16140 is considerable. A separate
ISO specification is being developed for the validation of in-house testing. However, this is
still in early stages of preparation and is not currently envisaged to encompass the validation
of methods for official testing purposes. The reference laboratory network considered the
validation requirements at the 2004 workshop and passed the following resolution:

7. Further to the above the NRLs agreed that it was necessary to define the appropriate
validation criteria for acceptance of alternative methods. NRLs consider that the
available ISO protocol ISO 16140 maybe too demanding for this purpose. The
workshop asked CRL and NRL attendees of the April 2004 Palma meeting of ISO/CEN
to raise this issue for discussion.

The matter was raised at the ISO and CEN meetings but no response was forthcoming. The
CRL was invited to submit comments to be considered when ISO 16140 is revised.

In this review of current microbial methods for the determination of faecal contamination and
resulting classification, no specific information regarding the validation of testing procedures
was received other than copies of the two listed validation procedures. An FVO mission to
France reported the used of a validated impedance technique and MPN method. Dupont et al.
(2004) describes the French performance of their MPN and impedance techniques (and other
impedance instruments under consideration). They are intending to start a revalidation of
their impedance technique against the ISO TS 16649-3 (Donovan MPN method) in 2005.

The Netherlands has reported that they are currently validating 4 methods, MPN old, MPN
Donovan, MacConkey plate count and Chromogenic agar plate count (Marnix P, personal
communication, 2004).
5.6 Proficiency Testing

Under Council Decision 1999/313/EC of 29th of April 1999, one of the duties of the CRL is “coordinating the application by the national reference laboratories of the methods referred to in point (a), by organising comparative testing in particular”. This comparative testing is principally undertaken through an EQA scheme run jointly by the CRL and the UK Health Protection Agency. This scheme is specifically intended for laboratories performing testing of live bivalve shellfish to meet the requirements of Directive 91/492. This approach was initiated at the 2002 workshop of the reference laboratory network and has been confirmed by subsequent workshops in 2003 and 2004. During 2003, the EQA distributions were supplemented with a ring trial distributed by the CRL to NRLs. The samples consisted of oyster homogenates. Such additional ring trials may be carried out in future years, depending on the outcome of further discussions at the workshops.

Under the same Council Decision, NRLs have the responsibility of “organising on a regular basis comparative tests between the various national laboratories responsible for the said analyses;”. In early 2004, the CRL conducted a survey of the extent to which NRLs were meeting this responsibility. Only 4 NRLs (Denmark, France, Italy, UK) reported performing proficiency testing among official testing laboratories for bivalve production areas. However, 8 of the 10 other NRLs which responded reported plans to introduce such comparative testing. Further to this, the 2004 workshop of the reference laboratory network passed the following resolutions:

18. The questionnaire on PT by NRLs in national laboratories highlighted the current low level of activity by NRLs - for example only 4 countries were carrying out PT for laboratories carrying out official control monitoring of bivalve mollusc production areas.

19. Further to the above it was agreed that all national laboratories carrying out official control monitoring of bivalve mollusc production areas should participate in a proficiency testing programme. It was agreed that this applied to all EU Member States having bivalve mollusc production areas.

20. Further to the above it was noted that 8 NRLs reported plans to initiate PT within 2 years. It was agreed that NRLs should report on the performance of testing laboratories at the annual NRLs workshop. It was agreed to consider and agree the confidentiality status of these reports at the next workshop.

5.7 Summary of Review

A number of different testing methods are currently being used in the classification of LBM production areas. The reference laboratory network recommends the use of the Donovan MPN method. From information gathered, not all of the ‘other’ procedures in use within the EU have been satisfactorily validated. Further information, in the form of translated copies of exact testing procedures employed by each country, would allow for a more in-depth comparison and review of testing methodologies. Initial analysis of the information provided to the Working Group indicates that further standardisation of testing procedures is needed (although there is some indication that more Member States are in the process of introducing the Donovan method). There is also a need to agree the minimum standard for validation of other methods and the minimum requirements for proficiency testing.
6. Numerical standards and analytical tolerance

6.1 Introduction

Live bivalve molluscs for direct human consumption must contain less than 300 faecal coliforms or less than 230 *E. coli* per 100 g of flesh and intra-valvular liquid (FIL) based on a five tubes three dilutions MPN test or any other bacteriological procedure shown to be of equivalent accuracy (Directive 91/492/EEC). This Directive establishes different categories in which a production area can be classified according to m value excepted for B zones where a criterion of tolerance is applied in the interpretation of the data (90% of the samples must present values < m). No information is given neither on the MPN test to use nor the interpretation of the results (measurement uncertainty, choice of the data set, etc.). Actually, all EU countries use an 5 x 3 MPN test (not the same) except The Netherlands (MacConkey plate count) and France (mainly 5 x 3 MPN test and impediometric technique for microbiological monitoring of production areas).

The Hygiene 3 Regulation establishes the same categories for *E. coli* without any tolerance criterion and sampling plan (European Communities 2004a). The draft regulation (EC) on microbiological criteria for foodstuffs (European Communities 2004b) fixes an ‘m’ value (< 230 *E. coli*/100g of FIL), the sampling plan (n = 1) and the analytical reference method (ISO TS 16649-3 - Donovan *et al*).

Most EU countries seem to follow strictly Directive 91/492/EEC except France, United Kingdom, Ireland, The Netherlands and Portugal which use criteria of tolerance in the interpretation of the data to classify production areas, i.e. a 3-class sampling plan decision criteria (see section 8.7.5. Appendix). France bases the application of tolerance criteria on the theoretical imprecision of the *E. coli* enumeration test.

The estimate of the value, i.e. regulation criterion, depends upon a great number of factors including the sampling plan, sampling procedures, method of analysis and culture media, etc. Without common and uniform specifications for the application and interpretation of regulatory criteria different Member States, or regional and local authorities, different decisions may be taken based on the same *E. coli* result. This section focuses on analytical issues of 5 x 3 MPN test, in particular the analytical variability in the interpretation of *E. coli* criterion.

Traditionally, results from microbiological analysis are presented unaccompanied by any form of uncertainty estimation. Microbiological tests of standard material are not available and the nature and behaviour of micro-organisms makes it difficult to apply to microbiological analyses the principles used for chemical analysis (NMKL procedure 1999).

6.2 Imprecision of *E. coli* enumeration

Under the hypothesis of a random distribution of bacteria in the test sample, *E. coli* bacteria are supposed to conform to Poisson distribution so that the numerical MPN standards are based on this assumption. In fact, bacteria in the marine environment usually show a log-normal rather than a Poisson distribution (Ashby & Rhodes-Roberts 1976). Using a Most Probable Number test the “true” *E. coli* number in a sample is never known (it should also be borne in mind that a count on an agar plate is also only an estimate of the true number in a sample). The detection limit is one bacterium or one positive tube (dilutions 1/0/0) for the
5x3 MPN test, i.e. one bacterium in 5.55g for each tubes is inoculated with 1g of flesh and intravalvular liquid. The *E. coli* number is calculate indirectly from the number of positive tubes using a mathematical formula, then De Man tables give results per g. (MPN coefficient 0.2) and the final result is expressed per 100g according to the regulation.

The MPN estimator is in a matter of fact the estimator of the maximum of likelihood (Beliaeff 1992). “Probable” in *Most Probable Number* has a different meaning from the one in probability theory. The “most likely bacterial density” would fit better as method name with regard to the distribution theory.

For low contamination levels the MPN leads to a overestimate number of bacteria, e.g. the relative bias is about 35 % on average for the 3x3 MPN test (Beliaeff & Mary 1993). Factors such as the absorption of bacteria to particles, as in bivalve mollusc homogenates, may result in the distribution of bacteria not conforming to a Poisson distribution, and the real count will be underestimated. Even after homogenisation of the sample, food of heterogeneous nature may have a heterogeneous distribution of micro-organisms (NMKL 1999). In such cases a negative binomial distribution will give a better fit (Trousselier *et al.* 1989).

A single MPN result has a low precision and most standards warn the user as follows “It is well known that wide variations in results may occur with the MPN technique. Results obtained with this method should therefore be used with caution”. When the decision to be taken on the basis of the result is of great importance, only category 1 result (95% confidence limits) should be accepted in preference to categories 1 and 2. Examples based on regulatory thresholds of Directive 91/492/EEC and Regulation (EC) nº 854/2004, are given in table 1 for 1 g (De Man 1983).

Table 1: Examples from MPN table for 5 x 1 g (ml), 5 x 0.1 g (ml) and 5 x 0.01 g (ml)

(Draft ISO TS 16649 – 3 Donovan *et al.*).

<table>
<thead>
<tr>
<th>Number of positive results</th>
<th>MPN index</th>
<th>Category when the number of samples (per batch) tested is</th>
<th>Confidence limits</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>0</td>
<td>2.2</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>0</td>
<td>2.7</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>0</td>
<td>2.3</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>1</td>
<td>3.1</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>0</td>
<td>3.3</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>1</td>
<td>4.6</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>0</td>
<td>4.9</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>1</td>
<td>7.0</td>
</tr>
</tbody>
</table>

1 When the number of micro-organisms in the sample is equal to the MPN found, the results is one of those that have the greatest chance of being obtained. There is only at most 5 % chance of obtaining a result that is less likely than the least likely one in this category.

**WARNING** The confidence limits given in Tables 1 are meant only to provide some idea about the influence of statistical variations on results. There will always also be other sources of variations, which may sometimes be even more important.

Therefore with regard to table 1, the 95 % confidence intervals vary from:
- from 70 to 660 *E. coli* for an MPN of 230 *E. coli* per 100 g of FIL
- from 1400 to 11300 *E. coli* for an MPN of 4600 *E. coli* per 100 g of FIL
- from 14000 to 113000 \textit{E. coli} for an MPN of 46000 \textit{E. coli} per 100 g of FIL.

Tillett (1987) contended that the use of confidence intervals for MPN estimations on samples from potentially non-homogeneous populations was not appropriate and that most probable ranges should be used instead – these give the range of results which are at least as 95% as likely as the MPN value itself. For an MPN value of 200, the most probable range is 180-220. Tillett also proposed that numerical standards based on MPN methods should be expressed in a form that took the characteristics of the method into account.

Two MPN estimates can be compared using the exact Fisher test (Beliaeff 1992). In case of 5x3 MPN test, the tubes selection 2/0/0 (45 \textit{E. coli} per 100 g) is not different from 5/0/0 (230 \textit{E. coli} per 100 g) with a 95% confidence interval. Such tests can be performed with impedimetric technique which uses parallel results but not with a single routine MPN. It should be noted, however, that results obtained using alternative methods such as the impedimetric technique, being calibrated against the MPN, will incorporate elements of variability due to the latter procedure.

6.3 Expression of results

Many laboratories meet difficulties to select the appropriate three consecutive dilutions in accordance with the standard rules because the explanations are not obvious. These issues often arise when proficiency testing is performed. Moreover the explanations lead sometimes to erroneous selection of the three consecutive dilutions. Beliaeff (1992) gives a simple rule for the different MPN tests, based on the true estimate, by example for the three tubes:

- One dilution gives at least 3 positive tubes : select the dilution the less high not giving 3 positive tubes and the 2 next dilutions. If this dilution give no positive tube, select the previous dilution and the 2 next dilutions.
  
  \begin{align*}
  3/3/2/1/0 & \text{ choose } 2/1/0 \\
  3/2/3/1/0 & \text{ choose } 2/3/1 \\
  3/3/3/0/0 & \text{ choose } 3/0/0 \\
  \end{align*}

- No dilution gives 3 positive tubes : select the three dilutions the less high.
  
  \begin{align*}
  2/2/1/1/0 & \text{ choose } 2/2/1 \\
  2/2/1/0/0 & \text{ choose } 2/2/1 \\
  \end{align*}

Based on the previous rule, the only right result is the number 5 (2/2/0) : 2,1 \textit{E. coli} per g (210 \textit{E. coli} per 100 g) and the others are:

- example 1 (2/1/0) : but the final result is the same 1,5x10^2 g\(^{-1}\) (15000 \textit{E. coli} per 100 g),
- example 2 (3/0/0) : 2,3x10^2 g\(^{-1}\) (23000 \textit{E. coli} per 100 g) and not 2,4 x 10^2 g\(^{-1}\) (24000 \textit{E. coli} per 100 g),
- example 3 (2/2/1) : 2,8 g\(^{-1}\) (280 \textit{E. coli} per 100 g) and not 7,4 x 10^1 g\(^{-1}\) (7400 \textit{E. coli} per 100 g), but the dilutions 2/2/1 are in category 3,
- example 4 (3/0/0) : 2,3x10^1 g\(^{-1}\) (2300 \textit{E. coli} per 100 g) and not 2,4 x 10^1 g\(^{-1}\) (2400 \textit{E. coli} per 100 g).

Table 2: Examples of selections of positive results for calculation of the MPN for three tubes

(ISO TS 16649-3 Donovan et al.)

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The use of an approved software package would be one way to reduce errors arising from the interpretation of the combination of results from several dilutions. Computer programs have been written to calculate the likelihood function, the MPN estimate of the density (Poisson and binomial models), its bias, standard error and confidence limits (Poisson model), for each combination of results (Beliaeff & Mary 1993).

6.4 Reporting of results with respect to their measurement of uncertainty

The report to the standing committee on the food chain and animal health (SANCO/0064/2003 – rev. 3; Anon. 2003a) recommends that “Enforcement Authorities shall use the measurement uncertainty associated with an analytical result when deciding whether an analytical result falls within the specification or not for food and feed control purposes. The way that measurement uncertainty is to be used by Enforcement Authorities must be taken into account when analytical specifications are discussed”. One of the issues, still under discussion with the experts of the Member States, is whether the measurement uncertainty should be taken into account for interpreting the conformity of test results to statutory limits and in what sense.

In practice, when considering a maximum value in legislation, this report recommends that “the analyst will determine the analytical level and estimate the measurement uncertainty at that level. The value obtained by subtracting the uncertainty from the reported concentration is used to assess compliance. Only if that value is greater than the maximum level in legislation, it is sure “beyond reasonable doubt” that the sample concentration of the analyte is greater than that prescribed by legislation”.

Since ISO 17025 has been published (ISO 1999), accreditation bodies requires that laboratories identify the sources of uncertainty for test results and make a reasonable estimate of the uncertainty validated associate with the results. According to the draft ISO TS 21748 “Knowledge of the uncertainty of measurement results is essential to the interpretation of the results. Without quantitative assessments of uncertainty, it is impossible to decide whether observed differences between results reflect more than experimental variability, whether test items comply with specifications, or whether laws based on limits have been broken. Without information on uncertainty, there is a real risk of either over- or under-interpretation of results. Incorrect decisions taken on such basis may result in unnecessary expenditure in industry, incorrect prosecution in law, or adverse health or social consequences” (ISO 2003b).
However, there are problems with applying analytical uncertainty to standards which have been derived without the explicit consideration of this issue as the resulting interpretation will either be stricter or laxer than that previously taken when uncertainty was not considered. Ideally, standards should be reviewed and reworded so that analytical uncertainty can be applied, preferably on the basis of a risk assessment.

ISO/TC 34/SC 9 *Food products – Microbiology* favours to define a general approach for quantitative determination based on the standard-deviation of reproducibility on the final result of the analysis. This approach prescribes to determine one (or several) value(s) of measurement uncertainty per target micro-organisms. The draft of the guide “*ISO TS Microbiology of food and animal feedings stuffs – Guide to the expression of measurement uncertainty for quantitative determinations*” have been circulated for comments (ISO 2003c). Assays performed by the beginning of 2004 accordingly this global or “top-down” approach met issues to determine the measurement uncertainty for solid and heterogeneous sample. A high level of laboratory effect was highlighted and probably a step-by-step approach would be required which represents a heavy work from each laboratory engaged in an accreditation procedure.

With reference to the uncertainties linked to the proposed microbiological criteria for foodstuffs in the document SANCO/4198/2001, the expert group agreed at its meeting of 26 March 2003 that the traditional approach of estimating an analytical tolerance of 3m quantitative limit as a common value for any limit controlled with a colony-count technique was out-dated. The expert group recommended following the ISO/TC 34/SC 9 general approach and gave its preference to the option “inter-laboratory standard-deviation of reproducibility” established through an inter-laboratory proficiency trial in which the laboratory has taken part. Measurement of uncertainty is calculated as twice the standard-deviation of reproducibility and when attached to a result is expressed as \(a+/−b\), where \(a\) is the result and \(b\) is the measurement uncertainty.

### 6.5 Analytical tolerance

In the report of an EU Working Group on Measurement Uncertainties (SANCO/58/003, WD 13.05.2003/PM; Anon. 2003b), the uncertainty determined by the Community Reference Laboratory (CRL) was given as \(+/-0.6\) log and to about \(+/-0.8\) log at low levels \(<100\ E. \text{coli}/100\text{g}\) near the nominal limit of detection of the MPN test \((20\ E. \text{coli} /100\text{g})\).

In order to ensure a high level of public health protection the regulation presents a very low limit for live bivalve molluscs \((230 E. \text{coli} \text{ per 100g})\), compared to others food products, but from a statistical view, as seen above, there is no real difference between \(45\ E. \text{coli} \text{ per 100 g}\) and \(230\ E. \text{coli} \text{ per 100 g}\). The 95 % confidence intervals of the value 45 vary from 7 to 280, which is above 230 (Table 3).

Based on Poisson distribution, French order of 21 May 1999 allows a 10 % level of tolerance among 12 monthly results, i.e. one value between 230 and 1000 \(E. \text{coli} /100\text{g}\) and no result above 1000. The 95 % confidence intervals (MU \(+/-\ 0.6\) log) of the nearer value from De Man table, i.e. 1100, vary from 280 to 4400. The NMKL procedure (1999) and the expert group (SANCO/0064/2003 – rev. 3) recommend that for a result to be considered as having exceeded a limit, the lower limit for confidence interval is required to be above this value.
Table 3: Regulatory thresholds and 95% confidence intervals with a MU +/- 0.6 log

<table>
<thead>
<tr>
<th>Measurement Uncertainty</th>
<th>≥ 95% confidence intervals</th>
</tr>
</thead>
<tbody>
<tr>
<td>MU +/- 0.8 log</td>
<td>3 &lt; 20 &lt; 125</td>
</tr>
<tr>
<td>MU +/- 0.8 log</td>
<td>7 &lt; 45 &lt; 280</td>
</tr>
<tr>
<td>MU +/- 0.6 log</td>
<td>58 &lt; 230 &lt; 910</td>
</tr>
<tr>
<td>MU +/- 0.6 log</td>
<td>1100 &lt; 4600 &lt; 19000</td>
</tr>
<tr>
<td>MU +/- 0.6 log</td>
<td>11000 &lt; 46000 &lt; 180000</td>
</tr>
</tbody>
</table>

The report to the standing Committee on the food chain and animal health (SANCO/0064/2003 – rev. 3, annex 1) gives illustrations of the effect of measurement uncertainty with regard to the interpretation of results against regulatory limits (see Figure 6.1). All Enforcement Authorities would take the decision without any hesitation in the following situations:

- when the analytical result together with the measurement uncertainty exceeds the statutory limit (situation 1) or,
- when the analytical result is less than the maximum value by an amount greater than the measurement uncertainty (situation 4). Applying this condition with a measurement uncertainty of +/-0.8 log, the only value met in De Mann 5x3 MPN table is 20 E. coli per 100 g.

In situation 2, the analytical result (for example 78 < 310 E. coli/100 g < 1200), exceeds the class A maximum limit (i.e. 230 E. coli/100 g) by less than the measurement uncertainty. Some Enforcement Authorities would ignore the measurement uncertainty and so would not accept the result while others would accept the result as being compliant with the regulation.

In situation 3, the analytical result (for example 52 < 210 E. coli/100 g < 830), is below the maximum limit by less than the measurement uncertainty. In general, Enforcement Authorities would consider the result to be compliant with the regulation.

![Figure 6.1. Interpretation of analytical results with respect to uncertainty](from (SANCO/0064/2003 – rev. 3, annex 1))
6.6 Conclusion

As emphasized in the report SANCO/0064/2003 (Anon. 2003a), “It is essential that interpretation of analytical results is similar if there is to be equivalence across the EU; […] It is stressed that this aspect is not a sampling or analytical issue as such but an administrative problem which has been highlighted as the result of recent activities in the analytical sector…”. It is intended that this matter will be addressed in the Good Practice Guide in lieu of progress across all product sectors.
7. Data handling and storage

7.1 Introduction

On the basis of the information provided to the working group by Member States, there appears to be great variation in the content of monitoring programmes with regard to the undertaking of shoreline surveys, available information on the location and nature of contaminating sources, information on the production of harvesting areas (producers and bivalve mollusc production), use of written records and databases. Different authorities often undertake different parts of the programme.

Prior to the classification and monitoring of bivalve mollusc harvesting areas, a shoreline survey seems of high interest to determine the sampling plan for each area (see precedent paragraphs 2.4, 2.5 and 2.6), even if this requirement is not stipulated in Directive 91/492. More details are included in the European regulation (854/2004). As a matter of fact, such an approach is taken into account in most of Member States (France, Greece, Ireland, Italy, Portugal, Spain, The Netherlands, United Kingdom,….) and in non-EU countries (USA, New-Zealand, Canada,…). Based on the actual practices and information used, a check list of useful information could be set up and guidance given on ways of recording such information (written records, databases). Annex 1 give examples of the various types of information collected by Member States.

7.2 Databases

Limited information is available on databases systems used in Member States, except for the United Kingdom and France:

- France: the data are stored in Ifremer database Quadrige (software Quadrige) which is a Sybase system running in a client server environment (Windows system) between each laboratory or personal computer (modem link) via the Renater (X25) network used by all French scientific institutes.

- The Netherlands: the data are stored in an Excel database by RIKILT and RIVO and re-entered and stored in database Board of Fish. Data are retrieved by the industry via the web maintained by Board of Fish.

- Spain:
  - Galicia: the data are stored in a SQL server database by the Centro de Control do Medio Marino which run the database from data capture to the report.
  - Andalucia: the results are real-time reported via Internet by software made for this purpose.
  - Cataluna: the data are stored in a Microsoft SQL server database and reported to the Regional fisheries authority and Central government authorities every 6 months.

- United Kingdom
  - England and Wales: the Shellfish Hygiene System consists of a Microsoft SQL 7.0 database closely integrated with Mapinfo Professional 6.5 running in a Citrix client server environment and replaces separate Microsoft Access databases and Mapinfo workspaces.
  - Scotland: an integrated database and mapping system is under development.
7.2.1 CEFAS (UK) and Ifremer (France) database systems

These systems are intended to be easier to use than the separate systems, and to offer advantages given by integration and in purpose of quality assurance of results and traceability from sampling to data storage. It is the basis for the management of the bivalve molluscs classification-monitoring programme.

The CEFAS database currently includes information on continuous and intermittent discharges to saline waters in England and Wales and data from the microbiological monitoring programme. It also has the capability to include phytoplankton and biotoxin monitoring data and information relating to purification centres.

Quadrige, Ifremer database, includes numerous monitoring programmes of the marine waters:
- nuclear power plant: hydrology, phytoplankton, zooplankton, benthos, ichthyoplankton, crustacean and fish monitoring data;
- microbiological, phytoplankton and biotoxin monitoring data;
- heavy metals, chemical pollutants monitoring data.

Security features

In order to help maintain the integrity of the data held within the two systems, access is password protected and users are individually assigned read only or write permissions according to organisational need.

Sewage discharges

CEFAS holds a database of consented sewage discharges. Details of new consent applications and modifications to existing consents are received from the Environment Agency as part of a consultation process designed to take account of potential fishery (including shellfish hygiene) and other marine impacts. The first figure below shows part of the list of discharges held within the database while the second shows the types of discharge categorised within the system. Information is held on the location of each discharge, its physical characteristics and potential impact, together with details of the consenting process.
Information on discharge plants are available on the site web (http://www.rnde.tm.fr/) of the French Water Data Network (RNDE). Others information on continuous and intermittent discharges to saline waters is collected by the Units for the Quality of Coastal Waters (Maritime and Navigation Service).
Microbiological monitoring data

The microbiological part of the CEFAS database includes 4 main tables. These contain details about: production areas, sampling points, sampling authorities and laboratories, results of microbiological testing together with other sampling information. The figures below show the screen by which details on the production areas and sample points are accessed, together with two aspects of the microbiological data entry screen. Most of the microbiological monitoring results for England and Wales are now received from the testing laboratories in electronic format and these can be automatically uploaded into the database.
The main structure of the database is shown in the figure below.

Quadrige Data

**POINT POSITION**
- SITE - HYDRO BASIN
- REGION
- DEPARTMENT

**STRATEGY**
- DATE
- PROGRAMME
- POINT

**TAXON**
- TAXON LEVEL

**ANALYSIS RESULT**
- PARAMETER
- METHOD

**SAMPLE**
- LEVEL
- TOOL

**SAMPLING CAMPAIGN**
- TAKING
- HOUR OF TAKING ON SITE

**DATE**

**PROGRAMME**
- MATRIX FOR ANALYSIS

**RESULT**
- ENUMERATION
- ANALYSIS

**RESULT**
- FRACTION
- ANALYSED

**RESULT**
- LABORATORY

**RESULT**
- SAMPLE

**RESULT**
- PROGRAMME

**RESULT**
- TAKING

**RESULT**
- STRATEGY

**RESULT**
- DATE

**RESULT**
- TOOL

**RESULT**
- LEVEL

Quadrige Ifremer database includes three modules:

- Data reference (27 main tables): programme (see figure below), strategy, method, matrix, species, site, basin, point (see figure below), event, resource, unit of measure, laboratory. This module is updated by the coordinator of the microbiological network.
• Data capture (8 tables): taking, sample (see figure below), result (see figure below), event (see figure below), closed site, checking, validation. This module is used by the laboratories producer of data.

• Data query (8 tables): result, closed site, event, strategy, parameter/matrix/method, region/site/basin, sampling point (see figure below). This module is used by all laboratory having a Quadrige software and password.

Data reference: Programme, code / point name, sampling lab, shellfish species (taxon)

Data reference: Sampling point information
Data capture: Matrix (bivalve), taxon (species) for laboratory sample

Data capture: *E. coli* result, method, laboratory and other information
Data capture: Example of pollution event

Data query: set of choice
Retrieval of data

In both CEFAS and Ifremer databases, data within all parts of the database can be accessed through forms, with filters applied as appropriate, or by standard queries. The retrieved data can then be exported to Microsoft Excel. Data can also be viewed in the form of graphs in the CEFAS database.

All microbiological data retrieved from Quadrige to SURVAL, database on the web site (http://www.ifremer.fr/envlit), can be retrieved in zip text files. SURVAL is updated each three months. Data are also retrieved by each Ifremer coastal laboratory (see the previous figure above) query and published in annual reports available on the web site.

Data audit

CEFAS database

There are two forms of audit within the CEFAS system. The first is that all edits to data in the system are tracked and reasons have to be given for such changes. The audit trail can be viewed by authorised staff. The second form of audit applies to the microbiological data. A number of audit values are applied to the data entry form which either prevent the entry of invalid data or warn when the data being entered is outside set criteria. The audit also applies to the E. coli results, if these are outside certain limits for the class of area in which the sampling point is located, a warning will be given. Further to this, there is a system whereby the entered data must be checked by a senior member of staff before it is deemed to be valid.

There are 4 levels of validation status: ongoing (unchecked and not released); pending (under investigation and not released); validated (checked and released); waived (due to exceptional circumstances).

Ifremer database

There are three levels of audit:
1. Check of capture data: each laboratory checks its data in conformity with the microbiological results.
2. Validation: each laboratory validates its data, the date of validation is registered for each result. The data can no longer be corrected and all laboratories can access to these data using a password.
3. Qualification (4 levels of validation status):
   - 0 : Non qualified, automatic value for all data validated
   - 1 : Good, value for data non suspect
   - 3 : Dubious, value for data that cannot be qualified yet
   - 4 : False, value for false data without any doubt (access impossible to the original data).

The coordinator of the microbiological network is in charge of the qualification of all data from the microbiological monitoring of bivalve molluscs harvesting areas. Microbiological data dubious or false are not entered in the database because they are not acceptable by the responsible of the microbiological laboratory.
Integration with the mapping functions

The default screen of the CEFAS application is a map of England and Wales and this is shown below.

Information on geographically related items in the databases (sewage discharges, sampling points, etc) can be accessed via this screen. A map showing the location of such items can also be accessed from within the database functions of the application.

For France, the maps of production areas, classifications and other information are available on the site web (http://www.rnde.tm.fr) of the French Water Data Network (RNDE). Such information is also available for sewage treatment plants.

Web-based data publication

CEFAS data from the microbiological monitoring programme is exported on a weekly basis to a web-site that can be accessed by the Food Standards Agency (the UK competent authority), sampling authorities and testing laboratories. This web-site also contains maps showing the location, extent and class of the production areas.

Results (graphs, annual reports), sampling points and maps, harvesting areas (description, classification) are available on the web site Ifremer http://www.ifremer.fr/envlit (see figure below), which is updated every three months.
Web site Envlit: Results of sampling point Port Groix from January 1989 to July 2004
Annex 1. : Examples of data required for the classification of production areas in EU

A.1. Greece
• Marine maps :
  - recording of industrial and urban waste water, discharge points,
  - recording of marine streams,
  - recording of rainfall,
  - production areas.

A.2. Ireland
• Map : map reference - boundary lines of production area - name of production area
  - location of shellfish beds - method of harvesting
• Site identification : site code - county - bay or area
• Sampling details : sampling point - nature of point - shellfish species

A.3. Italy
• Description of harvesting areas : nautical charts, description of the zone (map, classification, sampling points).
• Pollution sources :
  - map with location of the main sources of pollution (real or potential),
  - identification of discharge points and assessment of the pollution (domestic and agricultural unloading, industrial waste water, wild areas and stream),
• Hydrographical and meteorological characteristics : tide, rainfall, river mouths (seasonal volume) winds.

A.4. Portugal
• Location data : region – Port authority – harvesting area -
• Sampling details : sampling site – coordinates of sampling point - time and date of collection – tide – shellfish species

A.5. Spain (3 cases)

Andalucia
• Sampling details :
  - Location data : site identification and code – boundaries of production areas - sampling point coordinates
  - General data : collection date, time and method - shellfish species - weather – wind (direction and intensity) sea – tide - depth–secchi disk

Cataluna
• Sampling details :
  - Location data : map reference – site identification and code – boundaries of production areas natural beds (position and coordinates)
  - General data : collection date and time - shellfish species

Galicia
• Sampling details :
  - Location data : site identification and code – boundaries of production areas – sampling
point coordinates
- General data: collection date, time and method - shellfish species - weather – wind (direction and intensity) sea – tide - depth – secchi disk

A.6. The Netherlands
- Description of harvesting areas: maps (boundaries of the production areas and subdivision in sections), location of sampling points,

A.7. United Kingdom

England and Wales
- Sampling details:
  - Location data: CEFAS bed id - bed name - map reference (coordinates)
  - General data: collection date, time and method - shellfish species - water temperature

Northern Ireland
- Sampling details:
  - Location data: sampling site - bed name - map reference (coordinates)
  - General data: collection date, time and method - shellfish species – state of tide salinity - water temperature – wind direction and speed

Scotland
- Sampling details:
  - Location data: site identification and code – production area and boundaries map reference of collection point
  - General data: collection date and time - shellfish species – tide (ebb/flow – spring/neap) salinity - water temperature – turbidity - wind direction and force – rainfall (48 hrs) type of resource (shore, suspended or bottom cultivation, natural bed, other)
8. Interpretation of monitoring programme data

8.1. Introduction.

Under Directive 91/492, the data from the microbiological monitoring programme is used to determine the classification of a harvesting area (classification zone) according to the requirements given in Table 1.1 (the requirements of Table 1.2 will apply from 1 January 2006). The approach to the interpretation of data from the monitoring programme differs from that used for conventional microbiological criteria (e.g. ICMSF, 1986; Codex Alimentarius Commission, 1997. With bivalve mollusc harvesting areas the requirements relate to the area itself and not to individual samples/batches of bivalves and the data is used to essentially predict the risk of contamination of bivalve molluscs in an area by pathogens over the lifetime that the classification is in force. Interpretation of monitoring results in this regard is thus based on time series data sets. However, the important outcome of such assessments is the prediction of future risk and not the historical compliance in itself. Ongoing monitoring also determines whether the extent of contamination in an area has changed significantly and therefore whether short-term actions (e.g. closures) or a change in classification status is necessary. The monitoring programmes for the purposes of bivalve mollusc harvesting areas are thus more closely related to other environmental monitoring programmes than to assessment of the microbiological quality of batches of foods.

Neither Directive 91/492/EEC nor Regulation (EC) Nº 854/2004 stipulates the number of results (n) that are required to determine the classification or reclassification of a zone. They also do not include and consideration of tolerance that may be applied to results (other than the 90% compliance figure for class B under 91/492) or the minimum period over which results need to be obtained in order to detect variations in bacterial concentrations due to the influence of environmental factors.

The interpretation of the data of the programs (sampling plans) established for the classification and surveillance of the production areas must consider other factors such as: influence of environmental conditions, analytical variability, sampling points characteristics, sources of contamination and their characteristics, etc. The effects of these have been discussed in detail in earlier sections.

There are a large number of external factors, generally environmental (rain, state of the tide, wind regime, bathymetry, estuary circulation, etc.), that increase the variability of the environmental monitoring data. The effect of these external factors can be reduced using data sets containing large numbers of results obtained over time. This then avoids rapid fluctuations in the classification of the harvesting areas that would be the consequence when data sets are small or cover short time periods.

8.2 European legislation

Chapter I of the Annex of Directive 91/492/EEC establish the applicable conditions for classified production areas for bivalve molluscs production; regarding microbiological contamination levels detected, each production area can be classified as one of these three types:
- **Zone A.** Live bivalve molluscs taken from these areas must meet the requirements set out in Chapter V of the Annex of the Directive: areas must not exceed the limits of a five-tube, three-dilution MPN-test of 300 faecal coliforms per 100 g of flesh or 230 *E. coli* per 100 g of flesh or in any other method of bacteriological analysis of demonstrated equivalent precision.

- **Zone B.** Live bivalve molluscs from these areas must not exceed the limits of a five-tube, three-dilution MPN-test of 6000 faecal coliforms per 100 g of flesh or 4 600 *E. coli* per 100 g of flesh in 90 % of samples.

- **Zone C.** Live bivalve molluscs from these areas must not exceed the limits of a five-tube, three-dilution MPN-test of 60 000 faecal coliforms per 100 g of flesh.

Chapter VI of the Annex of Dir 91/492 indicates that the competent authority will fix a monitoring system of sanitary surveillance of the production areas in order to verify the fulfilment of the conditions established in the Directive as well as indicates the need to elaborate sampling plans that will have to consider, among other conditions, "the possible variations in the faecal contamination of each production or relaying area" although it is explained neither the required information to collect nor how this must be used.

Regulation (EC) Nº 854/2004 of the European Parliament and of the Council (Hygiene 3), due to be enacted in January 2006, establishes the same categories and limits for the classification of the production areas, with the only exception of specifying the use of *E. coli* but not faecal coliforms for the purpose of classification. This regulation establishes that, to classify a production area, the present sources of contamination in the area must be considered together with the characteristics of polluting agents contributing to the area and the circulation of them (See Section 2). Although these requirements are more complete and detailed than the established in Directive 91/492, the use of this information for the classification of the production areas has a highly subjective component in its interpretation and use. It is not defined neither the nature of the information to compile nor how this must be integrated in the dataset (the Commission has advised that these requirements will not apply to the zones currently classified, only those newly classified after 1 January 2006).

8.3. Interpretation of monitoring program data.

As a result of the lack of detail in the European legislation, mentioned in the introduction, there is a lack of uniformity in the way in which analytical results are used to determine the microbiological quality of a production area and, therefore, to classify it according to the parameters established in the legislation.

Table 8.3 contains a summary of some of the main aspects relative to the interpretation of the data of the developed monitoring programs in the EU Member States.

8.3.1. Minimum data set for classification and frequency of classification of zones.

The system of classification of the production areas established in the EU is based on the establishment of a monitoring system of sanitary surveillance that must be able to determine the underlying status of a production area regarding the identified levels of microbiological contamination and, at the same time, must have the capacity to determine any trends in such contamination. It is therefore fundamental that the data set used for the classification as well
as the frequency at which this classification is reviewed are sufficient to cushion the effect on the levels of microbiological contamination caused by other environmental or seasonal factors that can cause fluctuations or more constant changes in the classification of a production area. In the same way it is advisable to use a number of results that is statistically sufficient to be able to detect the inter-annual fluctuations in the sanitary classification of a production area.

Generally, the initial classification of the production areas in most of the countries of the EU was made from the historical data available not being habitual the creation of new production areas and, therefore, they do not contemplate in its protocols of performance the conditions that must be settle down for the classification of a new production area.

UK protocol establishes that a minimum of 10 samples is normally required from each identified monitoring point over a 3-4 month period before classification can be recommended for areas of class B and C, with samples being taken no more frequently than at weekly intervals. More monitoring data may be required for the designation of class A areas depending on the location of the bed and outcome of the initial 10 samples. At least 1 year’s worth of results with regular monthly monitoring are necessary for full classification status.

In Ireland, for classification purposes, each new site/production area must have twelve samples taken within a three month period. Classifications of all production areas are made twice a year, usually for the June to December period and the December to May period.

In France a "study of zone" is used to come to classify a new production area; in order to consider the phenomena of seasonal variability of the contamination, the "study of zone" is developed regularly, with a minimum duration of a year and, for the microbiological polluting agents, at least 26 measures for each sampling point are taken, with a minimum frequency of monthly sampling. A bimonthly frequency is generally used, except when it is not possible for technical reasons, in order to detect pollution events and seasonal variability. The samples are taken when the professional exploitation is maximum, that means during low tides of spring tides in case of Atlantic Ocean or Channel. For zones already classified, the analysis of the data is made on the three last years basis with the purpose of obtaining a set of results sufficient to detect the inter-annual fluctuations.

The Dutch and Swedish monitoring systems classify the production areas on the basis of each sampling occasion; in Netherlands data is assessed yearly for permanent classification status.

Lart & Hudson, (1993) establish that the most significant short-term factors affecting the level of bacteria in bivalve molluscs was found to be the state of the tide. In the longer term spring and neap tidal cycles were also found to be of importance and other seasonal factors, particularly freshwater runoff, also correlated with levels of bacteria in bivalve molluscs. These authors recommend that, in order to take seasonal factors fully into account, at least a full year’s data is required. There have been shown to be significant differences between neaps and springs; thus an equal number of neap and spring results are required. This could be accomplished by taking 26 samples spread over 13 or 26 lunar months; half of these would be at neap and half at spring tides.

8.3.2. Data set used in final assessments.
Diverse approaches are used in the EU in order to definitively fix or to review periodically the classification of a production area with respect to the accumulated data set, the nature of them and the form in which these are quantified:

The available historical data were used, in most EU countries, to undertake initial classification of the production areas.

In England and Wales, “Relevant historical data” is defined as being that covering the period for which the water quality in an area is believed not to have changed significantly i.e. there have been no significant changes to known inputs of contaminants. The formal review of the classification of a production area is made annually from the data of the monthly sampling comparing it with the classification of the previous year (ongoing assessments are also undertaken). If the classifications agree then these are accepted as “final”. If they differ, then all relevant data (relevant historical data and data from neighboring points) for the site in question is reviewed to determine whether the change in extent of contamination is significant. Relevant historical data, in addition to more recent data, can provide a better overall picture of the average underlying extent of contamination. This approach has endeavored to introduce an element of stability into the classification system in order to avoid the fluctuating classification scenario that may result from the consideration of only short-term datasets.

In England and Wales, some latitude is recommended in the criteria used for classification assessment if there has been historical compliance with the existing classification category. Similarly, if data from relevant neighboring monitoring points is fully compliant with the same classification category then this may also act as a mitigating factor.

In addition, the protocol for England and Wales establishes that, for a newly identified bed that is adjacent to an existing classified area, the addition of further monitoring points may not be necessary e.g. for an offshore bed with no contaminants inputs. In some instances, some parallel sampling may be requested between a point identified on the new bed and the nearest existing point. Should results from both points prove to be comparable after a small number of sets of samples then classification may be recommended at this stage. Monitoring would then normally continue at the existing point only. Should a difference between the results from each point become apparent then the new bed would be treated separately.

The Irish protocol take into account the results of samples taken and analyzed in the months or the previous year corresponding to the period being classified. The results of all other samples taken from the production area are also reviewed to include any additional relevant data in the consideration of any additional factors to be considered in the classification to be made. Where appropriate, additional measures are employed to assure the protection of human health from illness associated with viral contamination in bivalve molluscs. In particular, the consideration of any other risk factors that are present in or relevant to the bivalve mollusc production area being classified are incorporated in the classification decision.

Under the French protocol for classification (order 21 May 1999 and circular n°1607) values can be eliminated if they are clearly due to exceptional events as overflow or discharging of waste and if a solution can be found to avoid such consequences in the future.
In the case of results abnormally elevated with respect to the limits established for the classification of a production area it is necessary to make a detailed analysis in order to decide if the result must be considered for the classification of the production area or if it must not be considered; it solely seems recommendable to eliminate this type of values when they have its origin in an exceptional event, of definite temporal duration and with a minimum probability of repeating itself in the mid term; once finalized the effects of the event it is advisable to accomplishment a new sampling. The protocol for England and Wales establishes that results that can be attributed quite clearly to very unusual or “one-off events” that are unlikely to recur may be excluded from classification considerations. This will not, however, preclude the possibility of short-term control measures being applied to protect public health e.g. the issuing of a temporary prohibition order. Examples of events that may lead to results being disregarded are:

- Sewage treatment works failure.
- 1 in 5 year storm event.
- Failure to comply with the standard sampling protocol.

8.3.3. Estimation of microbiological quality in a production area

Directive 91/492 fixes the criteria to settle down the classification of a production area, based exclusively on the numerical values of microbiological contamination obtained in the monitoring programs (see 7.2). With exception of zones B (90% of the results), it is not established any criterion of tolerance in the interpretation of the data; nevertheless, the use of certain tolerance limits seems advisable in order to avoid excessive fluctuations in the classification of the production areas, specially those caused by accidental contamination incidents of short duration or those due to other external factors already highlighted.

Most EU countries follow strictly what is established in Directive 91/492 without using, therefore, criteria of tolerance in the interpretation of the data used to classify production areas. Other countries (France, England, Ireland, Holland, Portugal) use a different scheme for the classification of the production areas by means of using trigger values and levels of tolerance in the data, constituting a 3-class sampling plan decision criteria (see 7.1), contributing with M values (acceptability threshold) and c values. In the Appendix the tolerance levels used by UK and Irish, France, Portugal and Netherlands are described. The application of a system involving tolerance criteria seems advisable to avoid fluctuations in the classification of the production areas that, in fact, do not reflect real changes in the levels of microbiological contamination but the influence that on these levels have other external factors. Trigger values also provide the prompt to identify when the classification status for an area should be reviewed.

8.3.4. Criterion of stability of a zone.

IFREMER introduces in its monitoring system of microbiological surveillance (REMI control network) an interesting concept: "the stable or unstable character of a production area with respect to its microbiological quality".

The zones of "unstable" character are defined as:

- the zones A, B and C that have such a risk of degradation of the microbiological quality that they must regularly be reclassified A to B, B to C and C to Prohibited.
the zones B and C that have such an improvement of the microbiological quality that they must regularly be reclassified respectively to A or B.

The character of stability of a zone is defined according to the criteria present in Table 8.1:

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Validated</th>
<th>Character</th>
</tr>
</thead>
<tbody>
<tr>
<td>A estimated area</td>
<td>100 % of results &lt; 230 E. coli / 100 g of F.I.L. (tolerance : one value &gt; threshold)</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No</td>
</tr>
<tr>
<td>B estimated area</td>
<td>1st criterion : 100 % of results &lt; 4600 E. coli / 100 g of F.I.L. (tolerance : one value &gt; threshold)</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>2nd criterion : At least 20 % of results &gt; 230 E. coli / 100 g of F.I.L.</td>
<td>No</td>
</tr>
<tr>
<td>C estimated area</td>
<td>1st criterion : 100 % of results &lt; 46000 E. coli / 100 g of F.I.L. (tolerance : one value &gt; threshold)</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>2nd criterion : At least 20 % of results &gt; 4600 E. coli / 100 g of F.I.L</td>
<td>No</td>
</tr>
</tbody>
</table>

Table 8.1. Evaluation of the stability or the instability of a production area (in Document of prescription surveillance microbiologique. Cahier des spécifications techniques et méthodologiques REMI. IFREMER. 2004).

IFREMER establishes the sampling frequency applicable to a production area based on their microbiological quality (category A, B or C) and on the estimation of their stability (see Table 8.2). In agreement with the new requirements fixed in Regulation (EC) Nº 854/2004 relative to the control of contamination sources and environmental parameters, the criterion of stability or instability of a production area could be used to define different levels to control these parameters constituting itself as an element necessary to optimize the systems used for the classification of new production areas.

<table>
<thead>
<tr>
<th>Category</th>
<th>Character</th>
<th>Sampling frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>A estimated area</td>
<td>Stable</td>
<td>Quarterly</td>
</tr>
<tr>
<td></td>
<td>Unstable</td>
<td>Monthly</td>
</tr>
<tr>
<td>B estimated area</td>
<td>Stable</td>
<td>Bimonthly</td>
</tr>
<tr>
<td></td>
<td>Unstable</td>
<td>Monthly</td>
</tr>
<tr>
<td>C estimated area</td>
<td>Stable</td>
<td>Quarterly</td>
</tr>
<tr>
<td></td>
<td>Unstable</td>
<td>Monthly</td>
</tr>
</tbody>
</table>

Table 8.2. Sampling frequency according to the category (A, B or C) and stability of a production area
8.3.5. Interpretation of data in a “sampled area” with several monitoring points.

A production area must be homogeneous from the sanitary point of view or, at least, it must present a degree of uniformity such that, in "normal" circumstances, any sample of molluscs taken randomly in any point from the production are will present levels of fecal contamination within the limits established in the legislation for the class in which has been classified this zone. Therefore, it does not seem necessary to multiply the sampling points in the same production area; the only used point must be located in such a way that it allows to detect the changes that can be produced in the production area; it must be located in the sector of the production area more exposed to a possible polluting contribution; however, in case of very extensive zones or production areas with several sources of contamination, they must be used the minimum number of sampling points necessary to obtain a good representation of the zone.

The protocol for England and Wales explicitly considers the situation regarding beds with more than one monitoring point: the data from each point is considered and an overall compliance assessment made. Should one point show obviously worse contamination than others on the same bed, then the possibility of a split classification is considered. If a split classification is not justifiable or possible due to the enforcement problems that it would create, then consideration should be given to downgrading the entire bed.

In the Dutch protocol, sampling is performed by taking 5 samples of different fixed location in each area. For the evaluation of the results, a 3-sampling plan decision criteria is established for each sampling occasion (fixed values of m, M and c for class A; fixed values of m and M; in all cases n = 5); the classification of the production area is made with each sampling occasion.

The Danish Protocol specifies the taking of 3 samples of live bivalve molluscs within a specific area (3 positions) in order to establish a classified A area within a production B area. If the results of the analysis for E. coli / faecal coliforms are found under the legislative limits in all of the 3 parts of the sample set analyzed, then an A area will be opened.

The establishment of monitoring point buffer between zones of differing class can be considered, a priori, an important element in the establishment of a system of microbiological control; it presents the following advantages:

- Would allow to fix, with greater exactitude, the limits between zones of production of different microbiological quality and, therefore, of different classification.

- Would allow to detect the interchanges of microbiological contamination between both zones of production.

- It can be used as a warning system to identify the moment at which the classification of a zone of production must be reviewed.

Nevertheless, even though the concentrations of microbiological contamination undergo an important process of dilution from the pollution source towards the limits of the zone, this effect of dilution is conditional on the environmental conditions to such an extent that it is not possible to predict, with acceptable precision, the patterns of dispersion of the contamination. (Prokopenko et al., 1974 demonstrated that the number of sewage-associated bacteria
determined at two sites 3 km to either side of to sewage outfall were significantly influenced by the wind direction). For that reason, the use of buffer points in practice presents marked difficulties; among others:

- Difficulty to suitably select the position of the sampling point; it would have to be positioned exactly in the geographic position in which it is possible to detect the influence of the sources of contamination of both zones of production, independently of the prevailing environmental conditions.

- The introduction of greater levels of fluctuation in the interpretation of the data derived from the surveillance of each zone of production is foreseeable, especially if the buffer point is used in the determination of the microbiological quality of the zone of production (procedure of classification).

In order to be able to quantify the "border effect" between two neighboring zones of production with different microbiological classification the use of at least two control points could more be adopted, each located in the limits of the zones of production but in such a way that the underlying levels of microbiological contamination for each buffer point are sufficiently representative of the microbiological characteristics of the zone to which it belongs; it would be created, between both points, a land strip, more or less ample, foreseeably put under the influence of the conditions of both zones of production and with an ample fluctuation in the contamination concentrations.

8.3.6. Effect of environmental factors.

The classification of a production area primarily takes into account the results of the analysis carried out on the bivalve molluscs sampled from the production area but there are in addition a range of other factors that need to be addressed as being relevant when accessing the overall risk assessment of the area.

Reports in the scientific literature pertaining to the relationship between the levels of microbiological contamination and various environmental variables are numerous: Smith et al., 1999; Prokopenko et al., 1974; Krustulovic & Solic, 1991 identified wind as a factor that has significant influence in the movements of sewage-associated bacteria. Lart & Hudson, 1993, Catherine et al. 1995 and Wood, 1995 established that the tides are the main element that contributes to the distribution of the microbiological contamination. Belliaeff & Cochard, 1995 demonstrated that significant differences exist within the same production zone; this spatial heterogeneity limiting the interpretation of the influence of the different environmental factors.

These factors include the influences of: weather, particularly rainfall, the local topography, seasonal fluctuations in animal/human populations and their associated activities, water run-off and dispersal patterns, sea-water current patterns, tidal volumes and water exchange rates, the presence of foul water outlets, standard of treatment of human effluent treatment works, volume of output from these treatment works, frequency of output and the nature of the material being discharged, any intensive agricultural activity that could contribute to bivalve mollusc contamination, the species of bivalve mollusc being cultivated/harvested, the time of year when harvesting takes place, etc., the likely type of consumption of the shellfish being produced, raw, cooked, etc., the proximity of the shellfish beds to any of the above influences, any failures in sewage treatment plants or aspects of sewage treatment plant
design including overflow systems, method of treatment, etc., trends in the management of public health and the potential presence of viral contamination in the shellfish production waters.

These potential risk factors must be considered as part of the classification process. Nevertheless, most of these factors are difficult to quantify as themselves and this is still more complicated to quantify and to systematize their influence on the levels of microbiological contamination in the production areas; therefore, the knowledge of the contamination sources and the factors that contribute to change the impact of such contamination in the molluscs seems more useful for the establishment of the sampling points location than for their use in the classification of the zones. In the EU legislation, even though the use of these factors is contemplated, it is not described how to use this type of information with the object of classification of zones adding an element of high subjectivity to the process.

Therefore, even though the environmental factors as well as others must be consider for the classification of the production areas, the own nature of them makes any attempt of standardization very difficult in order to allow a uniform treatment between the EU Member States.

8.3.7. Additional classifications changes.

With the purpose of improving the levels of protection of public health, under certain circumstances, it can be advisable to make changes in the classification of a production area independently of the established system of classification review.

These changes can include modification of the boundaries of existing bivalve mollusc production areas, inclusion of additional species being harvested or the listing of new production areas that have been established since the most recent classification. This can be undertaken under both the Irish and UK systems.

8.3.7.1. Interim classifications.

In UK, if areas show sufficient deterioration or improvement in monitoring results during the year to warrant a downgrade or upgrade, or a new area is identified and sufficient monitoring data is available then an interim upgrade or downgrade is recommended.

8.3.7.2. Anomalous results.

The anomalous results are originated as a result of changes in the conditions of the production area, caused by natural phenomena (environmental) or caused by the action of the man; usually they have a definite temporary duration and, therefore, they must not change the observed tendency of the microbiological contamination of the zone except during a certain period of time; among other factors, they can be pointed out:

- Exceptional weather conditions, such as rainfall following drought conditions or periods of exceptionally heavy rainfall.

- Unusual agricultural activity in the form of slurry spreading onto the surrounding land.
- Failure in sewage management systems. When excess volumes of water are in the sewage system and have to be released as a storm overflow, or the waste treatment system fail or the infrastructure that carried sewage for treatment has failed.

- Sea borne pollution such as discharging of waste form seagoing vessels or marine leisure events increasing the biological loading in the water.

Remedial actions for the management of the anomalous results are different according to the different monitoring systems of the EU Member States:

- In general, an intensification of sampling in the affected production area is established; the collection of samples has to be made with, at least, one week of difference between consecutive samplings. This is in order to assist with the assessment of whether the underlying level of contamination has changed.

- Under the Irish protocol, local interests will be informed of any remedial action that may take place and may also place a prohibition order in the area if a threat to human health is apparent. Notification of a prohibition is also advertised in the local and national press, detailing the area that is being affected and the scope or the prohibition. Public health warnings may also be placed in and around the area affected.

- Italy, Spain (Galician and Catalonia) and the Netherlands (for downgrading an area from A to B) issue a provisional reclassification when anomalous results are detected that do not correspond to the limits required by the existing classification status; once the results are again in compliance with the initial classification of the production area, the provisional classification is rescinded.

- In Netherlands if values are found where 1/5 exceeds 6000 FC/100 g the area is normally not downgraded but closed for fishery activities. Where values were exceeded in the past this was incidental, the problems were always resolved within one or two weeks.

- For France, see Section 8.3.7.5.

8.3.7.4. Seasonal classifications

The protocol for England and Wales has the following requirements for seasonal classifications:

- At least 2 year’s worth of data showing a clear seasonal trend is necessary i.e. compliance with the upper classification category within the season and relevant in situ relay period as detailed below.

- The “active” season intended must be preceded by 2 months in situ relay period from class C to B (1 month from class B to A) i.e. the historical results during this period must also conform to the improved classification category.
In the Irish protocol, ideally the classification interval (twice a year, usually for the June to December period and the December to May period) should reflect the normal seasonal influences on the levels of contamination in the bivalve mollusc production area.

8.3.7.5. Alert monitoring procedures.

In France an alert procedure is established in order to intend the surveillance of non usual episodes of contamination or risk of contamination.

The alert procedures are established immediately in the following cases:

a) Exceedence or risk of exceedence of the values defined for each class of quality:
   - for a zone A, the alert level is 1000 $E. coli/100$ g.
   - for a zone B, the alert level is 4600 $E. coli/100$ g.
   - for a zone C, the alert level is 46000 $E. coli/100$ g.

b) Risk of contamination: meteorological event, polluting spill or information from third parties: Departmental Directorate of Health and Social Services alerts, Departmental Directorate of Veterinary Services request, shellfish industry information and others...

c) Outbreak of illness which is presumed to have been originated due to seafood consumption.

In cases a) and b), the alert is triggered immediately in a pre-alert stage. It is advisable to start, as quick as possible, a sampling in the set of sampling points of the production area and, as the latest, 48 hours after results for case a) have been obtained. Based on the results obtained in these analyses:

- if the result is below the level of establishment of the alert, pre-alert stage is finished.
- if the result is above the level of establishment of the alert, the pre-alert stage is confirmed. This supposes the establishment of a reinforced sampling.

In case c), the establishment of the pre-alert stage or the alert stage depends on the delay between the epidemic period stated or presumed with origin in the seafood consumption and the date of the communication of the existence of the presumed outbreak of illness with origin in the seafood.

Actually, the alert is translated essentially in the increase of the sampling frequency of the set of points of the production area. This frequency is weekly and it stands until the rise of the alert.

The alert stage ends after two consecutive series of results are obtained below the established levels for the establishment of the alert.

The results of the alert monitoring plan are sent as soon as possible by email or faxes to the list of local administrations (Departmental Prefect, Departmental Director of Maritime Affairs,...) in order to lead to regulatory measures for the protection of consumer health (bivalve mollusc purification, changing the status of areas, forbidding exploitation). The alert plan is immediately activated in case of storm overflow or sewage spill by example. All
information is sent to the local administration so that short-term control measures as temporary prohibition order can be taken to protect public health.

8.3.8. Interaction with environmental and analytical variability.

It is recognized that environmental monitoring data is inherently variable due to the effect of a number external factors (environmental factors) and variation in results may not be due to any significant change in the underlying extent of contamination. The effect of these influencing factors can be “smoothed” by considering larger dataset and, in this way, relevant historical data, in addition to more recent data, can provide a better overall picture of the average underlying extent of contamination.

At the present time, no EU Member State is explicitly taking the measurement of uncertainty, as determined by the testing laboratory, into account when determining compliance with the limits in Directive 91/492. The French criteria have been determined on the basis of the theoretical variability in the MPN test and these criteria are similar to those used elsewhere based on observed environmental variability (e.g. UK and Ireland).

8.4 Use of statistical tests

In France, two statistical tests are used to determine if differences exist in the contamination level between the sampling points of a production area:

- the Wilcoxon test when there are only two monitoring points in the production area,
- the Friedman test when there are more than two monitoring points in the production area.

A minimum data requirement of 6 series of parallel results for each sampling point in the harvesting area is specified. A redundancy between sampling points is suspected when there is no significant difference between the points. A single visual graphic analysis of the last six years of results for each point can often show if the sampling points show equivalent average levels of, and variability in, contamination.

The Mann Kendall test is used to determine the trend of the evolution of the microbiological contamination for each point. This test needs 10 years’ worth of data.
8.5 Discussion

The variation in sampling plans, sampling and testing approaches within the EU will all have an impact on the classifications that are determined on the basis of these. There are wide differences in approach to the length of dataset taken into account for determining classifications (from single samples to 3 years of monthly data) and the periods for which classifications were valid (2 weeks to 3 years). There are also vast differences in the number of “off samples” allowed before a revision or a downgrading of a classification is undertaken. Some member states have the procedure of “No tolerance is accepted, meaning no results over the limits”. Other member states accept that results exceed the limits such as 2 results > 230 E. coli but < 1000 E. coli, which may downgrade an A classification, and some “off” results are not taken into account if the cause of the pollution is identified. The basis for the establishing and interpreting data sets needs to be based on scientific evidence and a common approach needs to be undertaken across the EU.
<table>
<thead>
<tr>
<th></th>
<th>Normal length of the periods for which the classifications last</th>
<th>Length of “short-term control measures” “restrictions” or declassification to be implemented due to bad monitoring results</th>
<th>Number of “off “samples to cause revising and or downgrading of a classification</th>
<th>Use of “Short term” Increased sampling frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belgium</td>
<td>2 weeks.</td>
<td></td>
<td></td>
<td>Yes if analytical results requires. The frequency can be modified according to results.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denmark</td>
<td>All B- areas permanent. All A-areas open for 1 month – to keep it open requires monthly sampling. This will change to weekly by ultimo 2004.</td>
<td>Until a new set of 3 samples all are in accordance with the EU-limits</td>
<td>No tolerance is accepted. 1 of the 3 samples. This means that if 1 of the 3 samples analysed exceeds the EU-limits, then the classification is changed form A to B or form B to C. Exceeding the C-limits will close a production area for fishery or harvesting.</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>France</td>
<td>1 year: the classification may be reviewed each year</td>
<td>At least 2 weeks and until the previous situation returns. Temporary downgrading: 1 bad</td>
<td>The classification may be reviewed each year for all zones, based on routine</td>
<td>Yes to weekly (alert monitoring plan)</td>
</tr>
</tbody>
</table>

Table 8.3 Overview of temporal considerations – period of classification, short or long term restrictions
<table>
<thead>
<tr>
<th></th>
<th>Normal length of the periods for which the classifications last</th>
<th>Length of “short-term control measures” “restrictions” or declassification to be implemented due to bad monitoring results</th>
<th>Number of “off “samples to cause revising and or downgrading of a classification</th>
<th>Use of “Short term” Increased sampling frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Germany</td>
<td>No information available</td>
<td>result confirmed, i.e. 2 bad results.</td>
<td>survey results for the last three years.</td>
<td></td>
</tr>
<tr>
<td>Greece</td>
<td>1 year</td>
<td>Yearly decisions on status for classification based on the monthly analytical results.</td>
<td>No information available</td>
<td>No information available</td>
</tr>
</tbody>
</table>
| Ireland        | 6 months                                                      | A classification can be changed within the 6 months depending of results (dynamic classification) | For downgrading A areas: 1 result > 1000 E. coli or 2 results > 230 E. coli but < 1000 E. coli  
For downgrading B areas: 2 result > 18000 E. coli or 3 results > 4600 E. coli  
For downgrading C areas: 2 results > 46000 E. coli  
For upgrading B areas: 10 results < 230 E. coli over 1 year  
For upgrading C areas: 90% compliance with | Yes                                                          |
<table>
<thead>
<tr>
<th>Country</th>
<th>Normal length of the periods for which the classifications last</th>
<th>Length of “short-term control measures” “restrictions” or declassification to be implemented due to bad monitoring results</th>
<th>Number of “off “samples to cause revising and or downgrading of a classification</th>
<th>Use of “Short term” Increased sampling frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Italy</td>
<td>3 years as the minimum review period</td>
<td>Temporary measurements are lifted when a result after 1 week is in accordance with the EU-limits</td>
<td>No information available</td>
<td>No information available</td>
</tr>
<tr>
<td>Netherlands</td>
<td>All areas are A classified. If results equal B, then the area is declassified to B. After 1 week the area may again be reclassified to A</td>
<td>No information available</td>
<td>No information available</td>
<td>No information available</td>
</tr>
<tr>
<td>Portugal</td>
<td>Generally 2 years</td>
<td>If 1 sample exceeds limits then weekly</td>
<td>No information available</td>
<td>If 1 sample exceeds limits then weekly sampling</td>
</tr>
<tr>
<td></td>
<td></td>
<td>If 3 consecutive samples exceeds limits then declassified or closed.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Continued weekly sampling until 3 consecutive samples comply with limits - then reclassified.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spain</td>
<td>Galicia: 2 years, the calcification changes if 90% of results exceed limits in the last 12</td>
<td>Galicia, Andalucia: No information.</td>
<td>Galicia, Andalucia: No information.</td>
<td>Galicia: No information. Andalucia:</td>
</tr>
<tr>
<td>Country</td>
<td>Normal length of the periods for which the classifications last</td>
<td>Length of “short-term control measures” “restrictions” or declassification to be implemented due to bad monitoring results</td>
<td>Number of “off “samples to cause revising and or downgrading of a classification</td>
<td>Use of “Short term” Increased sampling frequency</td>
</tr>
<tr>
<td>-----------------</td>
<td>-----------------------------------------------------------------</td>
<td>-----------------------------------------------------------------------------------------------------------------</td>
<td>----------------------------------------------------------------------------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>Sweden</td>
<td>For the period following each sampling (5 subsamples)</td>
<td>Results exceeding limits a temporary declassification is put in place.</td>
<td>No tolerance is accepted.</td>
<td>Weekly sampling on secondary species. Cataluna: intensified sampling until the prior classification is back.</td>
</tr>
<tr>
<td>United Kingdom</td>
<td>1 year</td>
<td>Results exceeding the limits, originating from</td>
<td>Yes to every second week</td>
<td></td>
</tr>
<tr>
<td>Normal length of the periods for which the classifications last</td>
<td>Length of “short-term control measures” “restrictions” or declassification to be implemented due to bad monitoring results</td>
<td>Number of “off “samples to cause revising and or downgrading of a classification</td>
<td>Use of “Short term” Increased sampling frequency</td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>An interim upgrading or downgrading of a classification is possible depending on results. A special seasonal classification can be given for an area, if results over a 2 year period are in compliance with the upper classification category. Production areas with more than 1 monitoring point can get more than 1 classification.</td>
<td>very unusual event, may be excluded. For possible downgrading A areas: 1 result &gt; 1000 (E.\ coli) or 2 results &gt; 230 (E.\ coli) but &lt; 1000 (E.\ coli) For possible downgrading B areas: 2 results &gt; 18000 (E.\ coli) or 3 results &gt; 4600 (E.\ coli) For possible downgrading C areas: 2 results &gt; 46000 (E.\ coli) For upgrading B areas: 10 results &lt; 230 (E.\ coli) over 1 year For upgrading C areas: 90% compliance with 4600 (E.\ coli)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
8.5 Appendix

a) Trigger values and tolerance levels from UK and Irish.

From “CEFAS protocol for the classification of shellfish harvesting areas as carried out under the existing annual review process (2002)”.

<table>
<thead>
<tr>
<th>Class</th>
<th>Exceptional result</th>
<th>Cause for concern</th>
<th>Possible Downgrade</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1 result &gt;1000 \textit{E. coli}/100g</td>
<td>1 result &gt;230 but &lt;1000 \textit{E. coli}/100g</td>
<td>2 results &gt;230 and &lt;1000, or 1 result &gt;1000 \textit{E. coli}/100g</td>
</tr>
<tr>
<td>B</td>
<td>1 result &gt;18000 \textit{E. coli}/100g</td>
<td>2 results &gt;4600 but &lt;18000 \textit{E. coli}/100g</td>
<td>3 results &gt;4600 or 2 results &gt;18000 \textit{E. coli}/100g</td>
</tr>
<tr>
<td>C</td>
<td>1 result &gt;46000 \textit{E. coli}/100g</td>
<td>N/A</td>
<td>2 results &gt;46000 \textit{E. coli}/100g</td>
</tr>
</tbody>
</table>

b) Tolerance levels from France.


<table>
<thead>
<tr>
<th>Catégorie</th>
<th>Nombre d’Escherichia coli . (100 g C.L.I.) \textsuperscript{−1}</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>≥ 90 %</td>
</tr>
<tr>
<td>B</td>
<td>≥ 90 %</td>
</tr>
<tr>
<td>C</td>
<td>≥ 90 %</td>
</tr>
<tr>
<td>D</td>
<td>&gt; 10 %</td>
</tr>
</tbody>
</table>


c) Tolerance levels from Portugal.

From “TECHNICAL PROCEDURES. Live Bivalve Molluscs: criteria and microbiological limits of production or movement zones. Purification and dispatch centres: microbiological criteria and limits. IPIMAR,(2003)”.

- A Zones (limits: faecal coliforms ≤ 300 MPN/100g and no Salmonella in 25g)
  Occurrence of 1 sample of live bivalve molluscs outside acceptable limits:
  - intensify sampling, moving from monthly to weekly sampling
  - If 3 consecutive results are outside acceptable limits suggest to the competent authority provisional classification in the class corresponding to the figures obtained and continue with intensive sampling until 3 consecutive results are obtained that comply with acceptable limits for direct human consumption, at which point reapply classification A.
• **B Zones (limits: faecal coliforms > 300 and ≤ 6000 MPN/100g)**

Occurrence of 1 sample of live bivalve molluscs outside acceptable limits:
- intensify sampling, moving from monthly to weekly sampling
  ➢ If 3 consecutive results are outside acceptable limits suggest to the competent authority provisional classification in the class corresponding to the figures obtained and continue with intensive sampling until 3 consecutive results are obtained that comply with acceptable limits, at which point reapply classification B.

• **C Zones (limits: faecal coliforms > 6000 and ≤ 60000 MPN/100g)**

Occurrence of 1 sample of live bivalve molluscs outside acceptable limits:
- intensify sampling, moving from monthly to weekly sampling
  ➢ If 3 consecutive results are outside acceptable limits suggest to the competent authority the closure/suspension of the production area and continue with intensive sampling until 3 consecutive results are obtained that comply with acceptable limits, at which point reapply classification C.

d) **Tolerance levels from Netherlands.**

**Faecal coliform standards**

Faecal coliforms

- \( M = < 3000/100 \text{ g} \)
- \( m = < 300/100 \text{ g} \)
- \( n = 5 \) (number of samples)
- \( c = 1 \)

The results are positive when values exceed \( M \) and/or when \( c/n \) exceeds 1/5. The production/relaying area or compartments thereof can be downgraded to B or C areas depending on the extent to which salmonella and/or faecal coliform standards have been exceeded.

A areas are downgraded to B when

- \( M = <3000-6000/100 \text{ g} \)
- \( m = <300-6000/100 \text{ g} \)
- \( n = 5 \) (number of samples)

B areas are downgraded to C when

- \( M = <6000-60,000/100 \text{ g} \)
- \( m = <600-60,000/100 \text{ g} \)
- \( n = 5 \) (number of samples)

Downgrading an area to B status has happened for 1 week to a couple of weeks. Downgrading an area to C status rarely takes place; if values are found where 1/5 exceeds 6000 FC/100 g the area is normally not downgraded but closed for fishery activities. Where values were exceeded in the past this was incidental, the problems were always resolved within one or two weeks.
9. Discussion and conclusions

Information on Member State classification programmes was requested via NRLs following agreement on this matter at a reference laboratory network workshop. No formal questionnaire was produced as it was considered that the content of any such document would be based on the experience of the person(s) constructing the questionnaire and would prejudice receiving information on aspects of programmes that might be totally different to that experience. It was assumed that each Member State (or region) would have written descriptions of the programme content and formal protocols for many aspects such as sampling and laboratory testing. It appears that this is only the case for a proportion of Member States/regions and this complicated the receipt of relevant information. Relatively full responses were received from some NRLs, summary responses from others, with little or no response from two Member States. It was therefore not possible to undertake a full comparison of the approaches in all Member States and regions.

The review has shown that while there are a number of discrepancies between practices there are also a number of common approaches. For example, while there has been a wide variety of laboratory methods used for the enumeration of faecal coliforms or *E. coli* in the past, most Member States have either adopted or are in the process of adopting either the CRL-recommended Donovan method, or an alternative method validated against this. The two areas where there is greatest discrepancy are in the assignment of sampling plans and the subsequent interpretation of data. With regard to sampling plans, there is some agreement on the use of baseline monthly monitoring, with perhaps a reduced frequency for areas that are either temporarily out of use or have been shown to yield stable results. However, there are Member States that sample more frequently, usually allied to a shorter-term approach to classification status. With regard to the latter, there is a clear split between Member States that establish a longer-term classification based on time-series data and those that undertake a short-term approach to classifications, sometimes based around the results from single sampling occasions. It is sometimes difficult to determine which approach is being used, as it is not always clear whether all monitoring results from a harvesting area are taken into account when the baseline classification is reviewed or whether those results that determine short-term closures or downgrades are excluded from such reviews.

In considering progress towards a Good Practice Guide, it is important to consider not only the current practices undertaken within the EU, but also whether these are soundly based on the intent to protect public health and good scientific information. With regard to public health protection, there is a tendency to design and implement programmes to manage the faecal coliform/*E. coli* contamination and to forget the fact that there are significant differences in behaviour between these indicator bacteria and many of the pathogens of importance in causing bivalve mollusc-associated infection. In particular, there is a need to consider the way in which the indicator bacteria concentrations may vary greatly over the period of hours and that this may not represent the behaviour of the pathogens under the same circumstances. On the question of scientific knowledge relating to monitoring programmes (sampling plans, sampling and sample transport, laboratory methods, interpretation of data), the only significant information that was supplied to the working group came from France and the UK. Much of this work was done in the period immediately preceding the adoption of the Directive and up to the mid-1990s. While the scientific information from these two sources is valuable, there is a need to undertake further research in support of the application of the monitoring programmes.
Increasing the conformity of approach to the application of monitoring programmes would undoubtedly produce a more even level of public health protection and trade within the EU. However, for some Member States, it would impact on the viability of the industry, due to the consequent effects on classification status and might also increase the costs of the official programme.

10. Recommendations

1. The Working Group proceeds with the drafting of the Good Practice Guide based on the information received during the preparation of this review.
2. The contents of the guide should be based as far as possible on the principles of good public health protection and scientific information.
3. Additional research needs should be identified in order to improve the information available when the Guide is revised in future. This research will then need to be supported by the Commission and the Member States. Areas for research would include:
   i. Sanitary surveys: identification of practical approaches to determining the essential elements for each harvesting area
   ii. Relative spatial, temporal and species–specific variation in *E. coli* and pathogens using quantitative methods (Norovirus and Hepatitis A as viral pathogens; *Salmonella* as a bacterial pathogen).
   iii. Studies to support sampling and transport protocols
   iv. Methods for the analysis of monitoring data for the purposes of classification
4. All Member States should ensure that they have written procedures for the components of their monitoring programmes such as selection of monitoring points, sampling and sample transport, laboratory analyses and interpretation of data.
11. References


Godfree AF, Kay D and Wyer MD 1997. Faecal streptococci as indicators of faecal contamination in water. *Journal of Applied Microbiology* 83:110S-119S.

Hutchison D, Weaver RH & Scherago M 1943. The incidence and significance of microorganisms antagonistic to *Escherichia coli* in water. *Journal of Bacteriology* 45: 29


Galicia and Intergovernmental Oceanographic Commission of UNESCO; Santiago de Compostela, Spain. pp 253-264.


