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Bivalve Shellfish Hygiene Verification

Pre course study and support materials

Pre Course Reading Supporting the Seafish/REHIS Bivalve Shellfish Hygiene Verification Programme

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Part One of the course will introduce the purification process. It will then represent the Food Science and the Technology of the process as a platform of understanding for the carrying out of effective Official Control (OCs).

Principally, this is Approval and Inspecting which is the subject of Part Two of the course.

During the course reference is made to the following section on Official Control Verification (OCV).

OCV is a scientific, structured and systematic approach to Official Controls - Particularly inspecting.

Training in OCV in Scotland is through a 10 day training programme.

However, if you have not attended this programme, this will in **no way** disadvantage you on the shellfish hygiene programme.

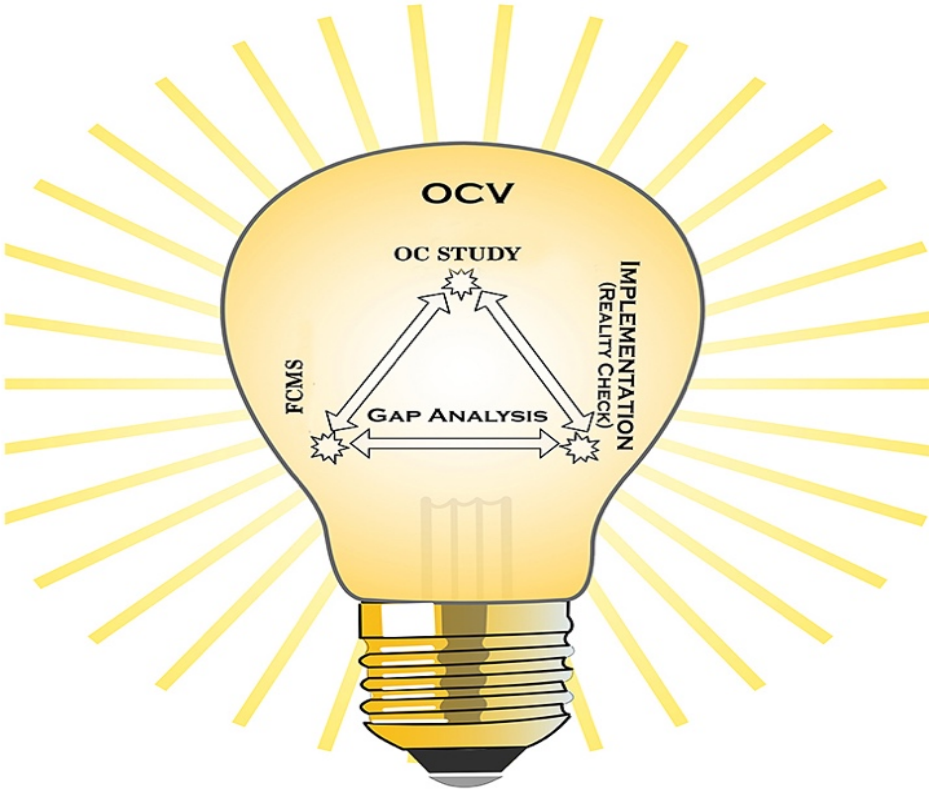
On the course we are discussing '**Triangulation**' and '**Evidential Triangulation'** as approaches to effective OCs applied to Shellfish Depuration. Studying the OCV material in this pack will prepare you well for this. The OCV HACCP Guidance (Section 2) should also be read, particularly pages 9 to 18.

The primer refers to papers which are also attached (appendices 1 & 2). It is recommended that you do not read these in full but read the abstracts and the conclusions.

We look forward to meeting you on the course.

Andrew MacLeod - Course Trainer

Section 1: A Primer on OCV



A Primer on OCV

1.0 Introduction

This primer is an introduction and an overview of OCV, according to its key principles. It also represents the challenges and the case for change that has led to the inception of OCV.

OCV is defined as a scientific, structured and systematic approach to Official Control (OCs) and is intended to provide the professions of EHOs, FSOs and Vets with a professional discipline for inspecting and all of the attributes of effectiveness , consistency and credibility that follow on from that position.

If you have not been trained in OCV you will not be disadvantaged during the shellfish Hygiene programme in any way. This primer will be sufficient by

representing the need for a scientific based professional discipline for inspections and the solution that OCV has provided.

1.1 A 5W 1H Analysis of OCV

What is OCV? - OCV is a scientific, systematic and structured approach to OCs. It was developed using deductive logic and in application it applies both deductive and inductive logic. At the heart of OCV lies a scientific and structured thought process (cognition) called Triangulation and a growing toolbox for its practical application. OCV is an integrated OC, applying to General Food Law, Food Safety, Food Hygiene, Authenticity (Food Standards) and to Food Fraud requirements. Thus, it pursues the integration of all aspects of Food Law into a single OC.

OCV **challenges everything**. FBO's propositions are never accepted. Instead they are subjected to independent scientific challenge by reference to **metrics**. The metric (measurement) of FBO performance are the requirements of Food Safety and Authenticity – Not Legal compliance. The metrics are not food law.

Enforcement of Food law is a separate activity downstream from OCV. OCV has been called a "*paradigm shift* "in the professions. OCV is a grass-roots approach in being designed by the professions themselves and takes an inspecting Officer's standpoint. This is a practical approach to realising high level Policy and Strategic goals, by bridging the gap between these high-level goals and the front-line professional practice of inspecting.

Why OCV ?- The headline here was to provide the various professions of EHOs, Food Safety Officers and Official Veterinarians with the attributes of a scientific professional discipline of their own. This pursues the Effectiveness requirement of The Official Controls Regulation (EU) 2017/625, creating statutory duties for all LAs and the FSS. This also pursues the scientific basis of Food Law established by the General Food law Regulation 178/2002. Consistency is logically a further outcome of a scientific based discipline.

When OCV?- OCV currently applies to all Approved establishments and to certain high risk non-Approved manufacturing FBOs in Scotland. A form of OCV with the current working title of '*OCV-Lite'* is expected for the catering and retail FBOs.

Who for OCV? - Currently all Officers who have passed the OCV training programme within LAs who have participated in the national OCV Pilot Project?

Where OCV? - OCV will be applied nationally to all Approved Establishments and certain high-risk manufacturing FBOs, as it becomes a statutory duty for all Competent Authorities when the FLCOP is revised. This is expected by 31st March 2022.

How OCV? - By application of the FSS/SFELC Guidance published in November 2019 and supported by the national training programme.

1.2 The Reception of OCV

Officers attending the courses have provided extensive feedback which has been overwhelmingly positive, with a recurring theme that the course is very intense, but frequently the best course that they have attended. The general evaluation descriptor has a score of 4.72 out of 5. Some Officers have been highly enthusiastic indeed and have rapidly become exponents of OCV.

OCV has been evaluated by EHOs, Vets, SFELC and scientists at the FSS. On each occasion it has been positively endorsed. The course of training now constitutes the majority of the FSS training budget.

The Scottish Government contracted '*Progressive*' a Company specialising in market research to further evaluate OCV. A range of stakeholders including industry, scientists and LAs were engaged within a process lasting 12 months. Similarly, the findings were overwhelmingly positive.

OCV is supported by the Minister.

Where the minority of feedback has been in contradiction, it has been in terms of the perceived complexity of OCV. However, when this perception was examined, it became clear that it is actually far more of a complex proposition, to attempt to justify continuing with inspections conducted in traditional ways. Engagement with Officers across the UK had confirmed the approaches taken were highly varied, inconsistently scientific, affected by subjective judgements, resource influenced, subject to political bias and equipped with very few tools by which to do the job of inspecting. In fact, far more attention has been paid to the administrative and accounting systems

(MIS systems such as FLARE and Uniform for example) than the actual job itself at the front-line. Literature review confirmed that the last UK publication on inspecting was actually in 1995.

Furthermore, clarification and reassurance that OCV is **scalable** in relation to the **Risk** and the **Complexity** of the FBO has led to further acceptance. This is represented in figure one below:-

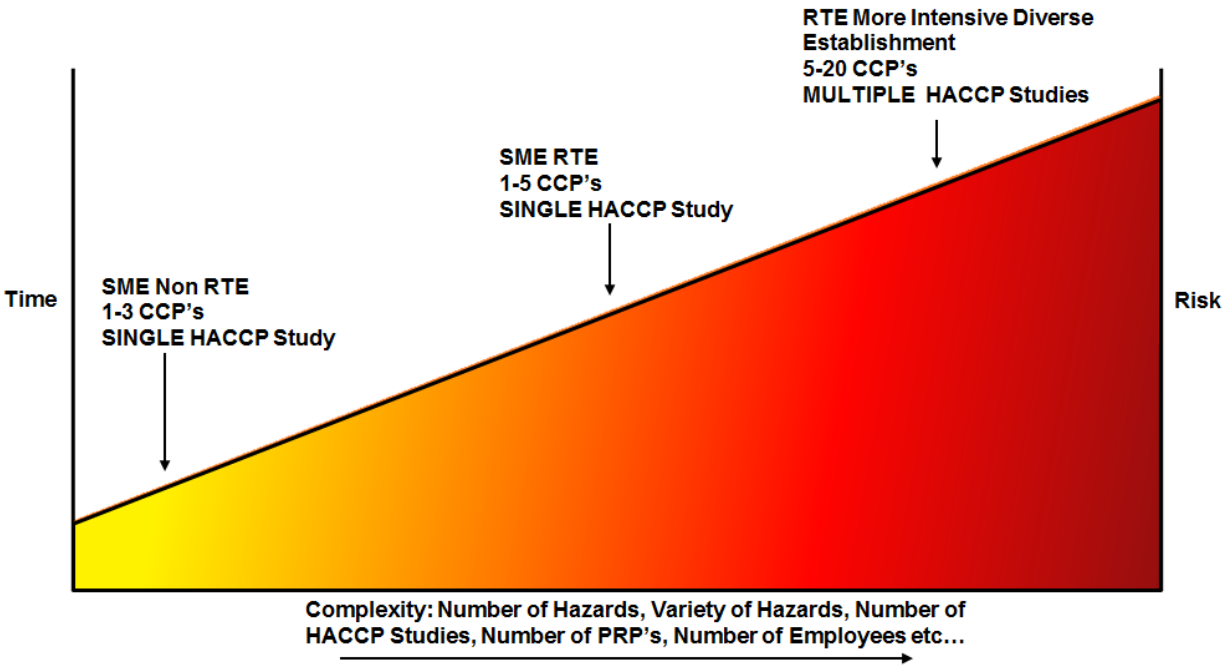


Figure One:- Scalability of OCV in Relation to the Risk and the Complexity of the FBO

OCV is based upon a fundamental principle called Triangulation (this is explained below). The extent and complexity of triangulation and the use of the various OCV tools, is always relative to the complexity of the FBO's establishment (e.g. number of products, number of HACCP studies, number

of process steps) and the risk of the process (e.g. RTE Foods and the hazards associated.

It is very worth keeping these points in mind when reading further.

1.3 The Drivers of Change

When advocating change, it is essential that the reasons for these changes are confirmed. There are several interrelated and overlapping reasons for the development of OCV, ranging from the aspirational to the hard realities of BREXIT. These drivers for change can be summarised as follows: -

- **Aspirational** – To give what other professions possess. The desire to provide the professions with a professional discipline for inspections and thereby to enhance the professional credibility of inspecting Officers.

- **Lack of efficacy** – Of legal compliance-based OCs. A body of literature (principally in the USA) confirms only a weak correlation at best between legal compliance and Food Safety outcomes. There is some confirmation of a similar position by the FSA for the UK. In addition, significant outbreaks of food borne disease have been attributed to large scale manufacturers that were actually fully compliant and indeed possessed numerous third-party accreditations. The literature also refers to weaknesses in the methodology of inspections. The nature of Food Law is intentionally designed to be '*generic*' and 'horizontal' in application. Logically that makes it a weak metric of FBO performance from an inspector's standpoint i.e. There is actually little detail to refer to in the regulations.

- **Lack of coherence** – Between Legal compliance-based inspections and the epidemiology of food borne illness, particularly with the causation aspects (i.e. aetiology).

- **Self Sufficiency -** The outbreak of E.coli STEC associated with raw cheese demonstrated that the professions required an approach to understanding non-standard and emergent Food Science and Technology. OCV provides such an approach.

- **Expert Legal and Scientific Opinion** – The FAI into the Wishaw E. coli STEC outbreak of 1996 has referred to *"a more educated scientific approach to inspections'*.

- **EU Food Law**: - The requirement for '*effective*' OCs in compliance with the Official Control Regulation. The General Food Regulation establishes EU Food Law on a scientific basis. Therefore, it logically follows that OCs need to be effective in scientific terms.

- **Consistency –** There had been recurring criticisms spanning 30 years from the multiple retailers and the industry that LAs took different approaches.

- **EU Veterinary Mission Audits**: - Successive EU veterinary Mission Audits have found weaknesses in UK OCs.

- **FSA Scotland's Focused Audits in 2009 and 20011 -** Found that HACCP systems were not being sufficiently verified by OCs.

- **Enlightened self-interest of the Professions and of EH Depts**
  Centralisation of Inspections of Approved Establishments was a '*die in the ditch'* issue for Charles Milne, former Director of FSA Scotland. This was prevented by a representation to the Minister by Argyll and Bute Council with East Lothian Council, in terms of OCV. Centralisation would have seen a significant loss of funding for EH Depts and a potential loss of the role for the LAs with all that entailed.

- **BREXIT –** It is widely anticipated that following BREXIT, Scotland's and UK OCs will be subject to further and potentially enhanced scrutiny from the EU auditors now called DG Santé F. OCs underpin the issue of Export Health Certificates (EHCs) and therefore adverse findings at audit have the potential to impede Scotland's and UK food exports to the EU. This is a political and economic driver for a change to OCs.

- **The Current Landscape of OCs –** Is undergoing a period of rapid change. For example, FLRS has integrated Food Authenticity and Integrity with Food Safety, and the SND is gathering data in what is the data age. However, most OC activities are all informed by and depend upon information gathered during inspections. If this information was wrong or incomplete, many other aspects would be adversely affected. Clearly, inspections play a strategic role in the broader landscape of OCs.

When these drivers for change are analysed, it is apparent that they all intersect at a science based professional discipline for OCs.

The following sections introduces and outlines OCV. It also outlines how it meets these challenges.

1.4 The Themes of OCV

Five overlapping themes underpin OCV as follows:-

- **Effectiveness** achieved by science (Service/ Professional efficacy)
- **Consistency** achieved by the common application of scientific methods and techniques.
- **Science requires metrics**. A metric is by definition a unit of measurement, In terms of OCV metrics of FBO performance. Metrics are objective and universally accepted. They are a fundamental principle of OCV and are the reason for the OC-Study (see below).
- **Simplification of complexity** - This requires that the purposes of inspections are understood, and to acknowledge that inspections actually amount to complex challenges that can and do overwhelm Officers. Furthermore, to appreciate a truth that before anything can be simplified, firstly its complexity must be acknowledged and understood. A major thrust of OCV is to unpick complexity into '*Bite-Size-Chunks'* which are manageable in terms of OCs, supporting the Officer in being in control and effective.
- **OCV is not a process** - It's the application of the Scientific methods to OCs.

1.5 The Role of Sampling

Before continuing it is important to explain that OCV does <u>not</u> require that every single aspect of an FBO's FCMS is directly verified according to the following principles. That is not considered an efficient use of resources.

Sampling of the FCMS is carried out. Here the FCMS in its broadest sense is referred to (e.g. Prerequisites Programmes, products and processes, HACCP Study and personal by interview), as the subject of sampling. This is not limited to sampling the food for laboratory analysis.

There are two forms of sampling:-

- **Elective Sampling -** where the Officer chooses to sample parts of the FCMS. This is used to ensure that those parts of the FCMS which are significant to the functioning of the FCMS, are high-risk in Food Safety terms and/or significant in Food Authenticity terms are verified.
- **Representative Random Sampling (RRS)** the scope of which can be any part of the FCMS. RRS requires that a representative portion of the FCMS is sampled and in sufficient quantities. From such a sample the attributes of the wider FCMS can be inferred.

Elective sampling and RRS are used in combination. This ensures that the most significant parts of the FCMS are verified in a representative way. For example, obvious candidates for Elective Sampling are Traceability, Hazard Analysis, Validation, Critical Control Points, and Provenance for example. The personnel involved in these aspects and the records for these aspects are examples of candidates for RRS.

Sampling the FCMS according to this discipline addresses the need to use resources efficiently.

The outcomes are that every part of the FBO's FCMS does not have to verified according to the principles outlined below. This is also very worth bearing in mind before reading further.

1.6 The Relationship Between OCV and the Scientific Method

This paragraph explores and outlines the relationship between OCV and the Scientific method. It is important to note that OCV does not seek to prescribe what to think - Instead it supports Officers in applying the scientific method.

Dr Neil deGrasse Tyson gets this over in Figure Two below.

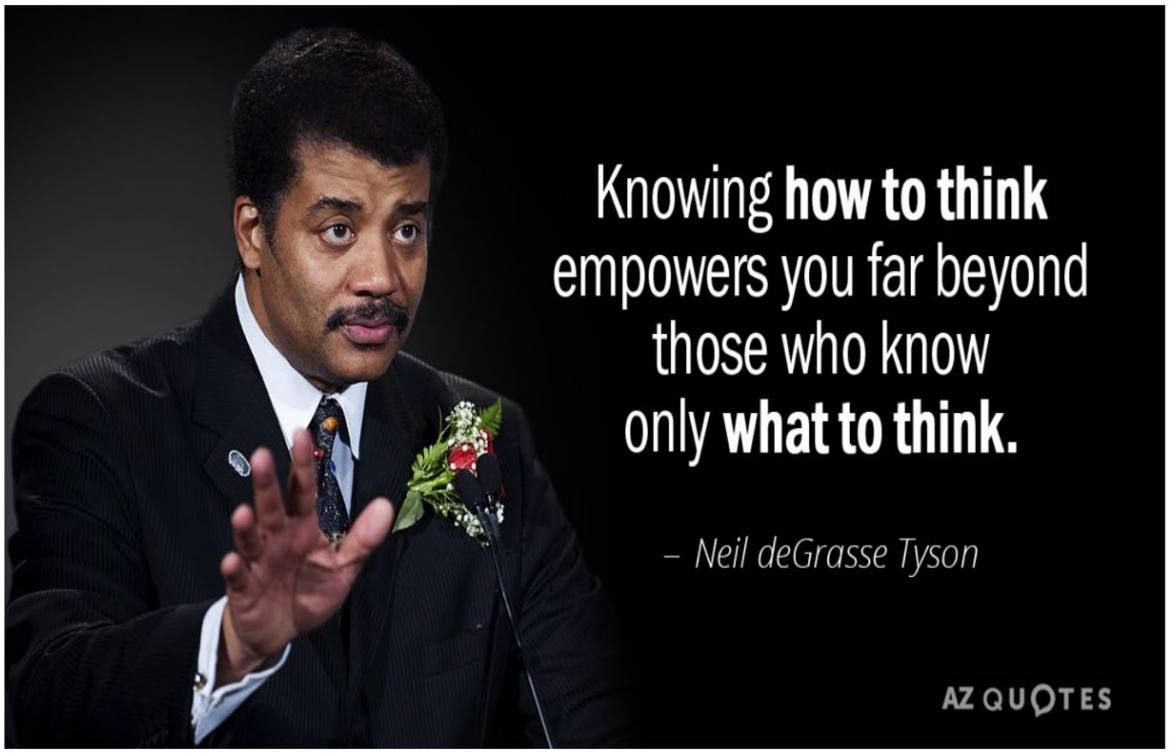


Figure Two - Dr Neil deGrasse Tyson on Thinking

OCV formally applies the Scientific method to OCs. For scientists this method is customary. For EHOs the lack of reference to **regulatory metrics** can at first feel counterintuitive.

Food borne illness is just the outcome of a natural process which starts in an unnatural environment – i.e. Food Processing. Food is colonised by saprophytes I.e. bacteria and fungi that break down organic matter. This natural process frequently has Food Safety implications. No matter how we choose to think or work – That truth is not going to change. Thus, we need to think scientifically which interprets Mother-Nature at work.

Furthermore, in Local Government and as Inspectors we do need to be careful that we don't fall into the trap of perceiving processes as how we should think or the point of that we do. They are not. Outcomes are the point of what we do and our thinking should be flexible and pursue our outcomes which is to verify FCMSs.

Deduction which originates with Aristotle and **induction** which is attributed to Sir Francis Bacon and to David Hume, are mainstays of the scientific method and of OCV.

This link is useful:

Deductive and Inductive Reasoning (Bacon vs Aristotle - Scientific Revolution) - YouTube

Deductive logic is reasoning from the point of an established truth, often called a premise or a priori. If the premise(s) is/are correct and the rules of logic are followed the answer is always correct. Deductive logic is often called reasoning like a mathematician. Exemplifications:-

'All swans are white, (premise). Therefore, the next swan I shall see will be white'.

"All FBOs must propose that the Food they place on the market is safe and is authentic (premises or propositions) ergo they must be eliminating or reducing the hazards to an acceptable level and the food is both as it must be and as it is described"

This is in fact the fundamental starting point of OCV.

When we refer to FBO's proposition, we are thinking in logic terms. We are deducing what the FBO must be doing if the food that they are placing on the market is both safe and authentic. We are not saying the FBO declares this out-loud or writes this down. Very rarely do they do this. We are fortunate to be provided with a comprehensive Product Description at Step 2 of HACCP in the larger food manufactures. It's a fact that most FBOs rarely think in explicit terms about what they are proposing, but just get on with it. In authenticity terms some of the FBO's propositions are stated on the label or the claims made about the food. However, many propositions are hidden. For example, if the FBO does not state that they are adding water - Then they propose that added water is absent from their product.

Inductive logic on the other hand is reasoning from the objective evidence. Reasoning from the evidence and following the rules of logic leads us to a conclusion that is the most likely to be correct. Inductive reasoning is often called reasoning like a scientist. Exemplifications:-

" All the swans that I have ever seen have been white. I conclude that the next swan that I see, will also most likely be white"

"I have interviewed Mrs. McCabe and she clearly understand the SOP. I have observed her and two others applying the SOP correctly. Ten random samples of the records indicate that the SOP is being applied. I conclude that the most likely explanation is that the SOP is being applied correctly"

The relationship between OCV, the Scientific Method, Deduction and Induction is shown in figure three below.

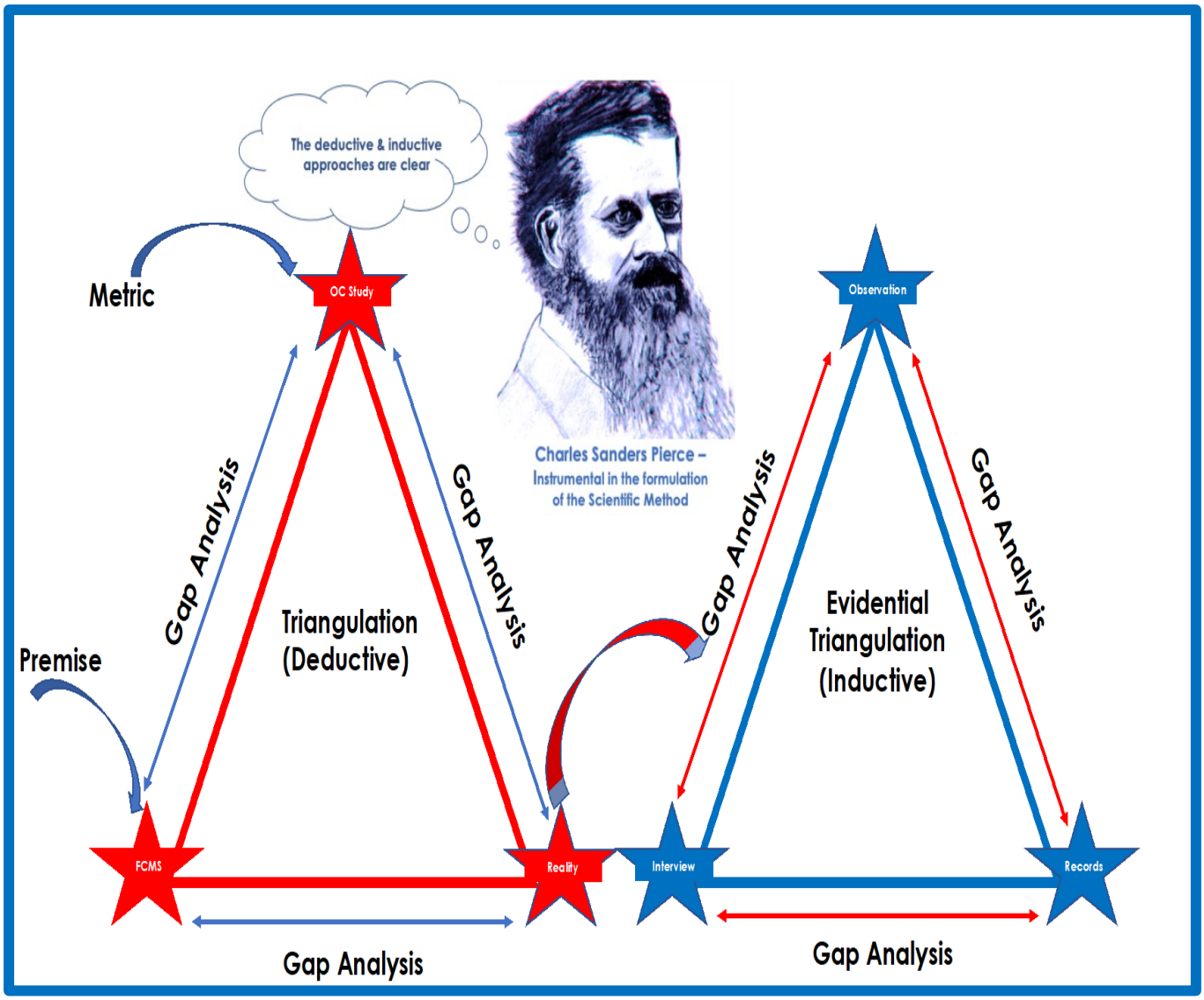


Figure Three:- The relationship between OCV, The Scientific Method, Deduction and Induction.

This is considered in further practical detail below.

2.0 Triangulation

Figure three above represents the scientific cognition (critical thinking of OCV) projected onto paper.

Reference is made to the right-hand side of figure three i.e. Triangulation (the deductive part).

This addresses the question:-

" ***Does the FBO intend to do the right things in the very first place?***

This question is given appropriate enhanced emphasis in OCV. To answer this critical question a scientist would require a metric of Food Safety and of Food Authenticity by which to compare and contrast with the FBO's own propositions. A scientist would never accept the FBO's proposition without testing it first – And neither must we!

The inspecting Officer compares and contrasts the FBO's FCMS with the OC-Study, which is a study carried out by the Competent Authority. This is totally independent of and discreet from the FBO's FCMS. This process is called **Triangulation** and **Gap Analysis**. This study comprises of a literature review on the **Food Science and Technology** and the **epidemiology** of the FBO's

process, a formal 12 Step WHO-CODEX based HACCP study and a literature review of the Food Authenticity aspects.

All the propositions that the FBO makes are potentially subject to the process of Triangulation.

Where the FBO's proposition triangulate (reconcile) with the OC Study, it is deduced that the FBO has been proposing to do the right things.

How this is applied specifically to Food Safety and then to Authenticity is the subject of paragraphs 2.1 to 3. below.

2.1 '*Shoe-Leather Epidemiology*' - Developing Our Metric of Food Safety

Experience has confirmed that a couple of examples from other areas of Public Health help to illustrate this principle.

Reference is made to figure four below. This is a spatial and temporal analysis of the airborne spread of COVID-19 within a restaurant carried out by Chinese Public Health Officers. Cases were associated with distance from the index case, time associated with the index case and with the pattern of airflow which has back eddies. It is noteworthy that the distances far exceeded 2 meters.

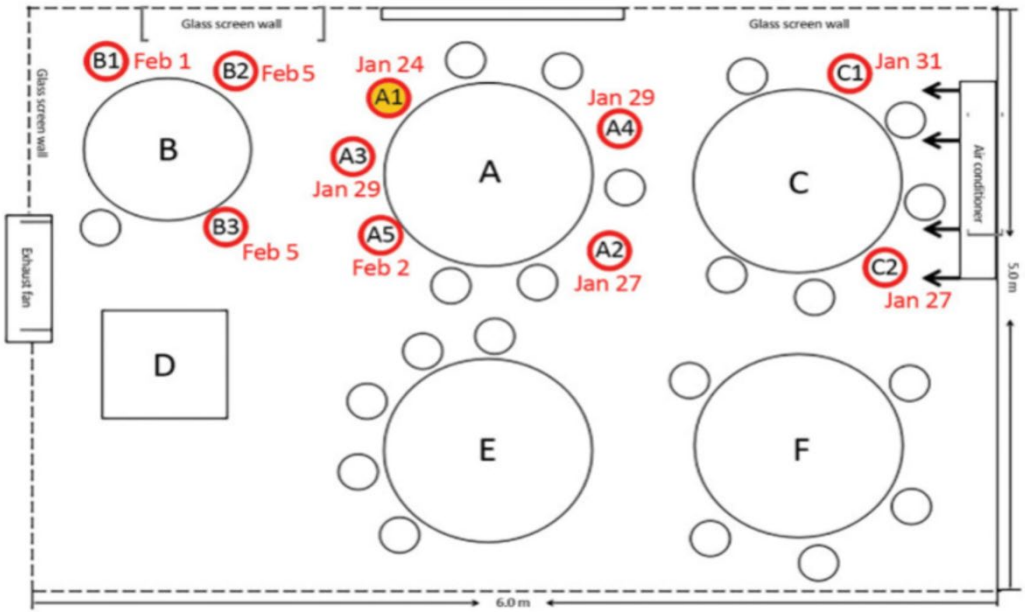


Figure Four:- Spatial and Temporal Association Between the Index Case and Secondary Cases with Distance, Time, Exposure and Air flow (A to D are tables & A1 is the index case).

Erin Bromage Associate Professor of Biology at the University of Massachusetts Dartmouth has called this

"*really great shoe-leather epidemiology*"

'*Shoe-Leather'* is an American metaphor for evidence based logical analysis at the front line - Doing the *'legwork.*

This little piece of epidemiology is a metric of the spread of COVID-19 in this and similar situations. If we wanted to prevent the spread of COVID-19 we would require this knowledge. If we wanted to hold FBO's and politicians to account in a scientific sense, we could refer to this work as a metric.

Probably the most famous example of this approach is John Snow's investigation into the source of Cholera in London. Before microbiology had

even confirmed the existence of microbes, using only logic, a yardstick and balls of string, John Snow was able to infer an association between 150 cases of Cholera and contiguity to a certain water standpipe in Broad Street, Soho which has become contaminated with *Vibrio cholera.* Reference is made to figure five below.

Figure Five - John Snow's Cholera Map. Water Pumps are Denoted by Blue Triangles and the Number of Cases by the Size of the Red Circles.

This is the first recorded piece of epidemiological investigation. It provides a metric of the spread of Cholera in these circumstances.

What is clear in each approach is a systematic science-based analysis of the pattern of the spread of disease. John Snow went on to use this knowledge as a metric by which to hold the Authorities and water suppliers to account.

OCV builds upon the very same logical and systematic approaches, but it is informed by this established epidemiology of food-borne illness

The first foray into this approach in Food Safety was the work of Diane Roberts and Dr Frank Bryan. This can be accessed through the attached papers. It is suggested that these papers are only scanned at this stage.

This work became highly influential, but sadly today is often overlooked. It became the mainstay of Hazard Analysis at Step 6 of HACCP. OCV has taken this work and further developed into the OCV metric.

A metric is developed in the form the pattern of foodborne illness within an FBO's establishment. The FBO is verified against this metric. OCV takes this approach during an OC-Study usually from the desktop and also during a Reality-Check phase whilst walking the line.

In practical terms the Officer develops the metric in the form of a **Hazard-Map**. Applying their Advanced (Level 4) HACCP skills to the FBO's process, the Officer carries out an independent Hazard Analysis (WHO-CODEX HACCP Step 6) of the FBO's process and propositions.

This is based upon the epidemiology of foodborne illness established by Dr Bryan and Diane Roberts which confirms a '**chain-of-events'** involving the **Hazards** and their ***Contributory Factors*** (i**.e., presence, Introduction by direct contamination, Introduction by indirect or Cross Contamination, Multiplication and Survival** summarised as **P.I.I.M.S** or **P.I.G.S**. ***Contributory Factors*** are a recuring concept in epidemiology. They amplify a hazard or enable the hazard to lead to illness - And importantly they can be used to associate a hazard to a step within a process flow diagram where to is relevant to do so. This is expalined below.

The FBO's process flow diagram is interpreted in terms of this chain-of-events.

This is carried out as follows: Hazards are associated with the particular steps in the process flow by identifying the most relevant ***Contributory Factor***. The Contributory factor places the hazard on to the process flow diagram at the relevant step (To confirm that is relevance here is in terms of the established epidemiology of food borne illness - refer above).

This is called **Hazard Mapping <u>and is one of the most powerful tools in all of Public Health.</u>** This is because when completed the Hazard Map is actually **<u>predictive</u>** of the chain of events that would lead to food borne illness, if the FBO was not applying Control Measures.

Figure Six below shows just such a generalised Hazard Map for the Chains of events leading to Food Borne Illness.

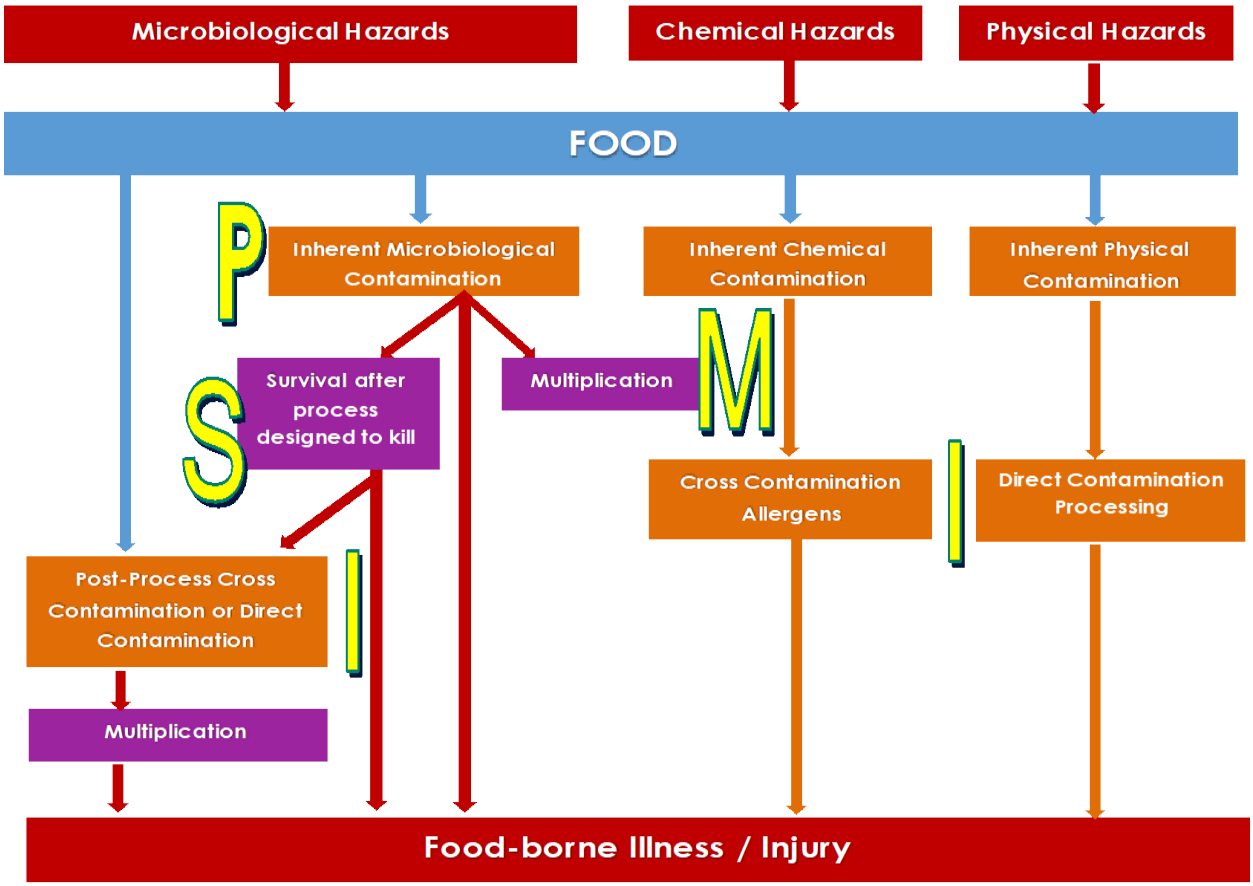


Figure Six - Generalised Map of the Epidemiological Chain of Events Leading to Food Borne Illness. The Contributory Factors (P=Presence, I = Introduction by Direct Contamination, I = Introduction by Cross Contamination M= Multiplication and S=Survival) Will Interact with the Hazard so as to Produce an Infective Dose in the Food - If Control Measures are not Applied by the FBO.

From this informed and predictive position, the Officer is able in terms of the epidemiology of foodborne disease to precisely hold the FBO to account for the correct Control Measures, in the correct places at the correct time. This map and this informed position are the **metric** by which the FBO is held to

account. The Inspecting Officer verifies that the correct Control Measures are being implemeted by reference to this **metric.**

When the OC-Study triangulates with the FBO's propositions all significant hazards have been verified as under control. The FBO is verified as safe.

Nowhere do the regulations provide the Officer with such an exact metric by which to hold the FBO to account. Epidemiology has now become the metric. OCs are clearly connected to the Effectiveness requirements of the Official Control Regulation and with the scientific nature of Food Law required by the General Food Regulation.

2.2 '*Shoe Leather Authenticity*' - Developing Our Metric of Food Authenticity

In Food Authenticity terms, the FBO's propositions are subject to the same general process. We conduct an OC Study again but this time in terms of Food Authenticity.

However, in this case the metrics are very different. In this case the legislation is much closer to a scientific metric.

Food standards legislation can seem quite complex and overwhelming. Certainly, there are a multitude of regulations. However, applying an OCV 5W1H approach it is clear that the legislation addresses just a few fundamental, overlapping questions:-

- Quantities of food or ingredients
- Qualities of food or ingredients

- Provenance of the food
- Information provided to consumers by FBOs (Note this is cross cutting with Food Safety)

This is very clear if we look at the Food Standards Training Manual published by the FSS/SFELC.

The metrics are as follows:-

- Quantitative prescriptions - e.g. absence of certain substances, amounts of permitted additives or ingredients etc.
- Qualitative prescriptions/qualitative claims, e.g. Protected names/provenance and FBO descriptions/representations etc.
- Provenance
- Information provided to consumers

Triangulation is carried out in the same way as above. This time referencing these metrics in the OC Study and of course the FBO's propositions.

3.0 Comprehending Complexity and Dealing with It

In practical terms the Triangulation approach outlined above is a start. That is all it is. It is not actually very useful in practice. This is because FBO's propositions are frequently very complex indeed. They in fact consist of numerous component propositions.

From this reality there are two practical problems for the Officer which can be deduced.-

- Complexity cannot be simplified until the complexity is firstly understood
- Trying to verify every component proposition at the same time is extremely difficult.

To solve this problem an OCV tool was produced. The tool is an OC tool called **Recursive Triangulation** which uses deductive logic in reverse and is an application of **Reverse Engineering.** Reverse engineering is the practice of systematically disassembling a product or a process in order to understand its overall design and the thinking that went into that design.

This link is a tele-visual representation of the Reverse Engineering concept:-

What does REVERSE ENGINEERING mean? REVERSE ENGINEERING meaning, definition & explanation - YouTube

Typically, the result is like an '*exploded-diagram*' of the internal workings of a product. In essence with the triangulations below we are now going to ''*explode*' what the FBOs propositions must be if the food is going to be safe and authentic. We can deduce in detail what the FBO must really be proposing if their propositions of Food Safety and Food Authenticity are true.

If we think again in these terms of the FBO's proposition of Food Safety, we begin to break it down. If the food is in fact safe, deductively we begin to see

that the FBO must be proposing Control Measures for every biological, chemical and physical hazard associated with the product and/or process in terms of every permutation of P.I.M.M.S. There can be hundreds of permutations. In one Advanced HACCP case study consisting of 46 process steps, the scope of which is confined to considering only the two hazards of *Clostridium botulinum* and *Listeria monocytogenes* then there are 512 permutations of events leading to food borne illness. The math is as follows.:-

*46 process steps X 2(hazards) X 4 (*P.I.M.M.S.) = 512

This is already a lot for one Officer to verify. We have not yet considered the other hazards. These will add many more to that number. The number increases rapidly as more hazards are considered.

As we begin the think about Food authenticity in the same way, we would begin to see that the FBO must be proposing Control Measures for aspects such as ingredient provenance, Master Manufacturing Instructions (recipes) and Food Information for consumers for example. The number increases much further.

Clearly trying to verify all of this at once would be overwhelming.

To make things still more complex, the FBO may well be proposing Food Safety and Authenticity in relation to a process that is new or unknown to us - And remember the FBO may not really understand all the real details of the requirements that they are proposing.

The Officer requires a tool that will map out the complexity of what the FBO is actually proposing and then break that down into '*Bite-Sized-Chunks'* which are manageable to the Officer and amenable to planning.

The basis of such a tool is called **recursion** - A form defined by its own form such as Serpienski's triangle in figure 6 below. The is a representation of triangulation of the overall proposition of the FBO, recognising that it is in fact comprised of numerous component triangulations as shown below.

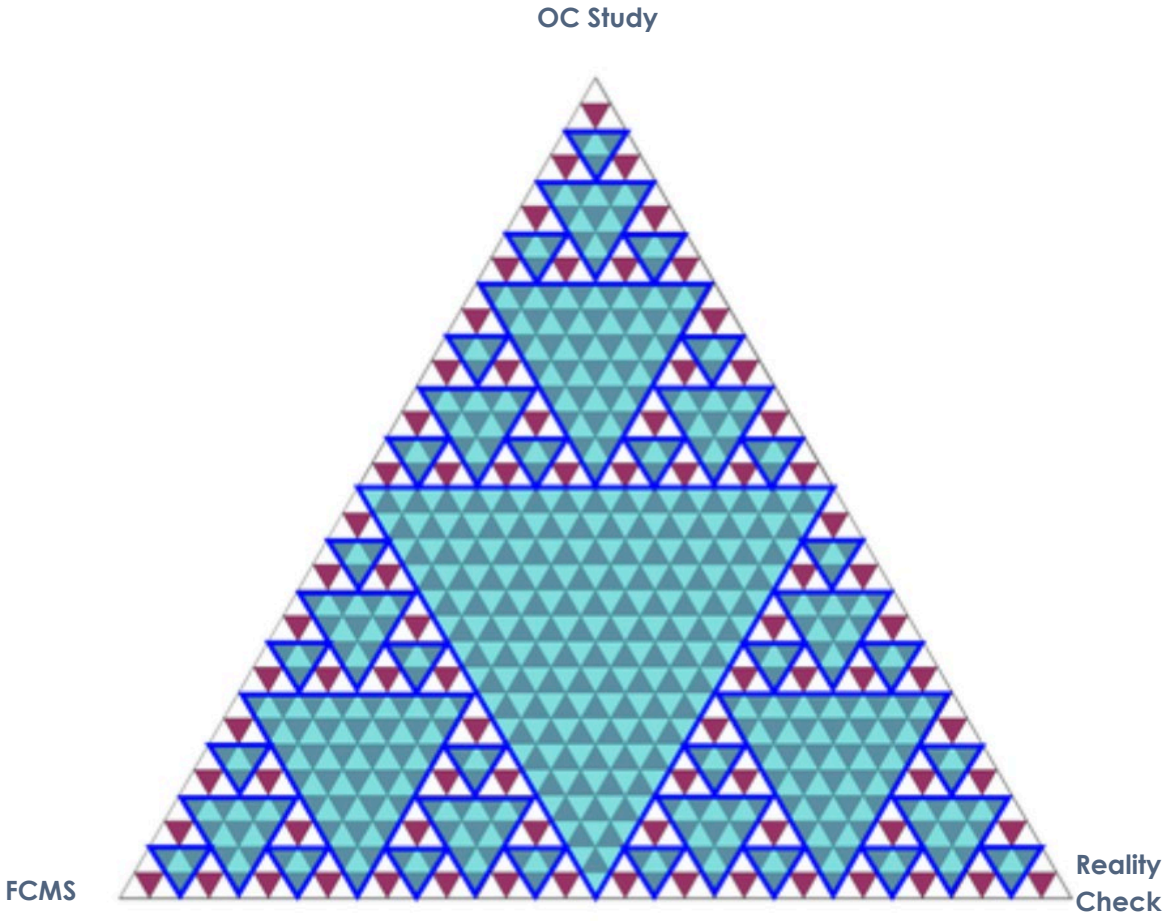


Figure Five - The Relationship Between the Overall Triangulation and its Component Triangulations

An FBO's proposition can be understood by successive iterations of deductive deconstruction of what that proposition consists of. There are three key outcomes to this:-

- An understanding of what the FBO's propositions actually are and revealing the actual complexity - (Remember complexity cannot be simplified unless the complexity is first understood).
- Simplifying the FBO's proposition into manageable '*Bite-Sized-Chunks'*
- A '*map*' of the verifications required which can be converted into an inspection form and an inspection plan if required.

This approach will be demonstrated and discussed through two examples, the first in Food Safety and the second in Food Authenticity.

3.1 Mapping the Triangulations - Food Safety Example

Reference is made to Figure Seven below.

This triangulation relates to a Coq Au Vin ready meal. This triangulation specifically focusses upon the control of *Listeria monocytogenes*.

It is important to imagine this as part of a much larger triangulation of all of the FBO's propositions - i.e. one of the triangles within figure Five above.

Workflow -Arrow of Deduction (Deconstruction)

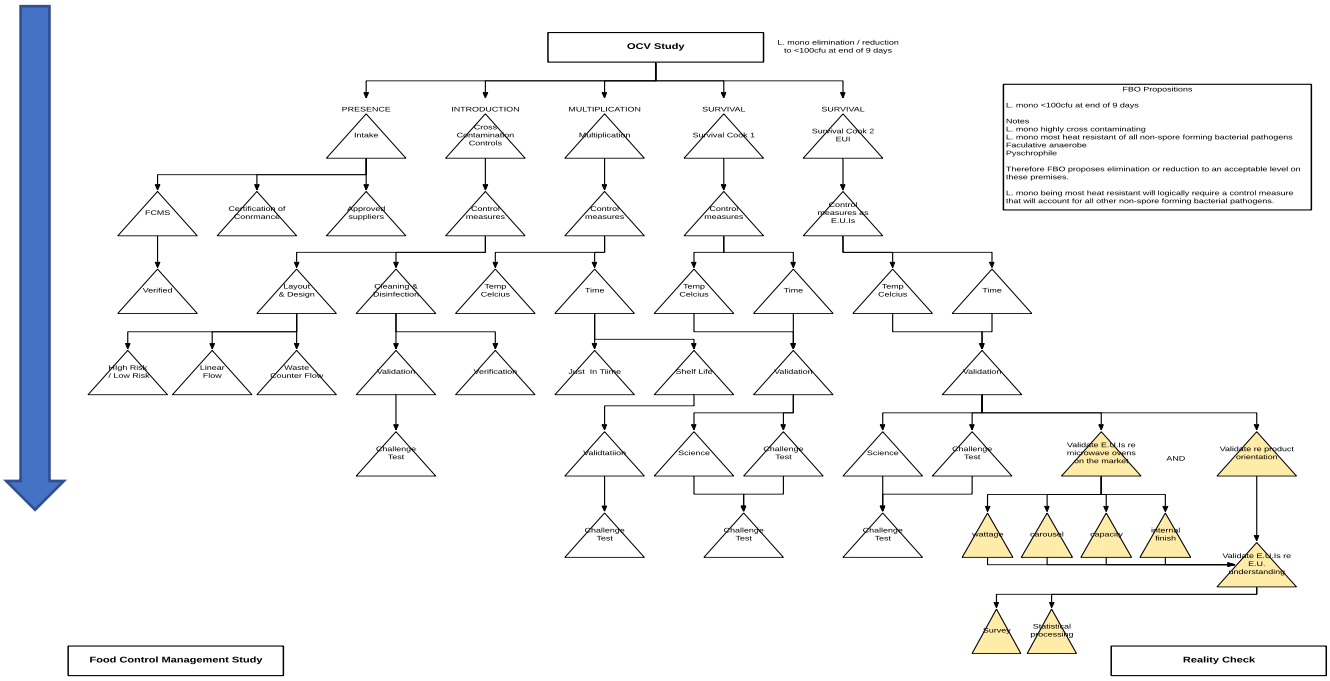


Figure Six:- Summary Triangulation of the FBO's Proposition for the Control of *Listeria monocytogenes* in Relation to a Coq Au Vin Ready Meal.

Figure Six can be understood as an '*exploded-diagram*' of what verifications of a sound FBO proposition on Food Safety would look like - Built by an Officer on the basis of deductive deconstruction.

The FBO's propositions are listed on the RHS. These propositions are derived deductively, from research into the food science and technology, the microbiology and the epidemiology of the product and process undertaken during the OC-Study. On the left-hand side is an arrow indicating the direction of the workflow which is the deductive process of deconstruction or break-down.

Computer software called Lucid-Chart has been used to assist.

The approach has been to deduce from the Hazard Analysis referring to the '*Contributory Factors'* (P.I.I.M.S). This approach breaks down the proposition from the epidemiology of food borne illness i.e. if the FBO is in fact placing safe food upon the market, then the FBO must be controlling the Hazrds and their Contibutory Factors (P.I.I.M.S). In practice propositions are broken down by successive iterations of deduction according to the following general pattern of thought:-

"If the FBO proposes 'A' then deductively they must have 'B' which deductively means they must have 'C' which in turn means they must have 'D, E and F'."

This continues until a proposition cannot be broken down any further.

The outcome is now a map of all the triangulations required to verify a proposition. This has been produced deductively and the premises are the established epidemiology of food borne illness. Therefore, this provides a map of an effective Official Control.

Complexity has been captured, represented and simplified. One triangulation can be undertaken at a time. This is the '*bite-Sized chunks*' approach.

3.2 Mapping the Triangulations - Food Authenticity Example

Reference is made to figure seven below.

This example relates to a Bouillabaisse (seafood stew) ready meal, where specific claims have been made in relation to the species of ingredients and their provenance.

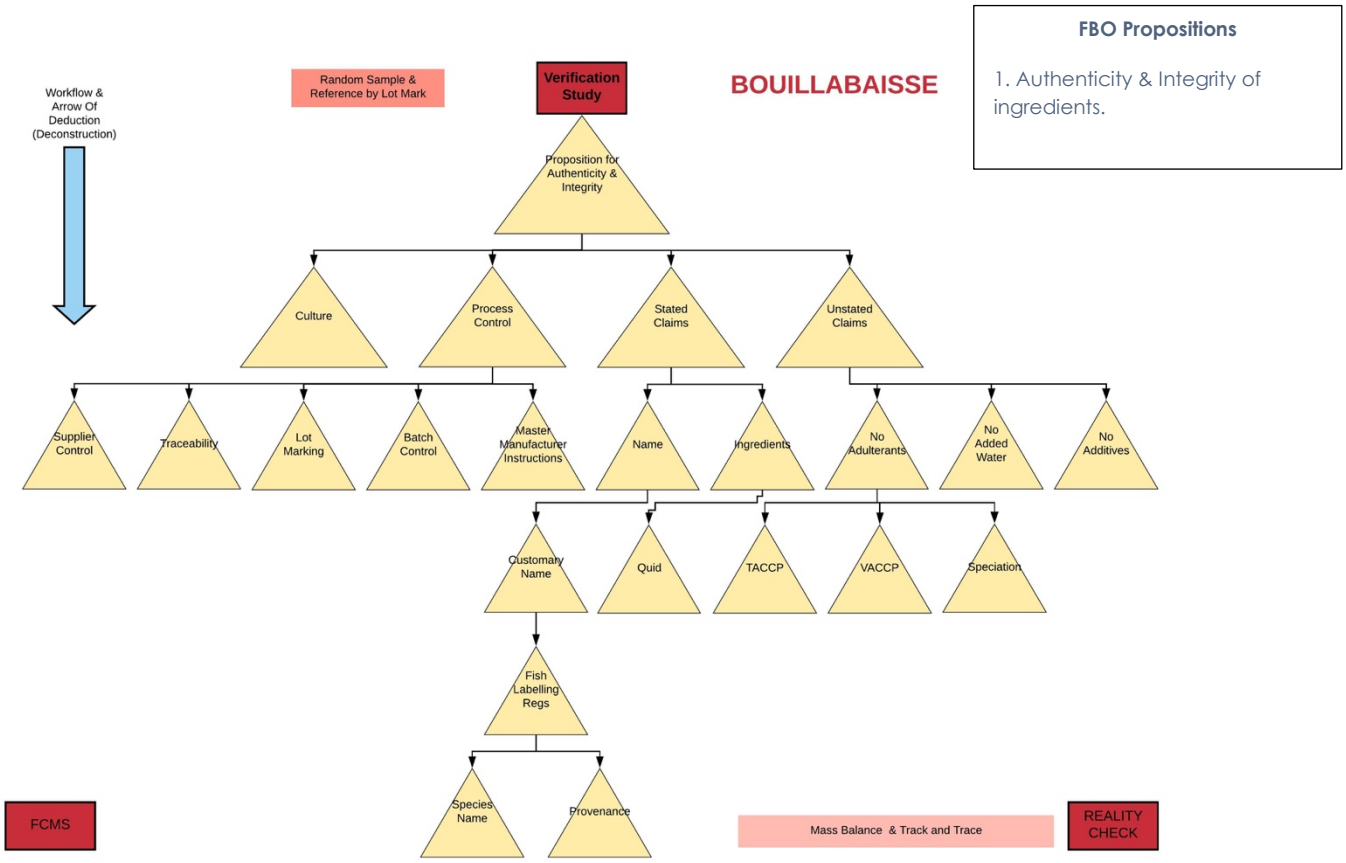


Figure Seven:- Summary Triangulation of the FBO's Proposition for Food Authenticity in Relation to a Bouillabaisse Ready Meal.

Similarly, on the right-hand side the FBO's propositions are listed. These propositions are derived deductively from research undertaken during the OC-Study. In this case this research includes, Food Standard legislation, the FBO's claims, the Food Science and Technology of the product and process. On the left-hand side is an arrow indicating the workflow which again is the deductive process of break-down

In practice the approach is the same with propositions being broken down by successive iterations of deductions as outlined above.

The outcome is a map of the all of the triangulations required to verify a proposition. This provides a map of the Official Control.

4.0 The Reality Check - Evidential Triangulation

The Reality Check is intended to answer the question:

"Has the FBO actually been doing the things that they have proposed to do?"

In a similar process of Triangulation and Gap Analysis this question is addressed. In this case the reference points are different sources of **Objective Evidence.**

Work in the humanities (i.e. studying human behavior) is strongly supportive of triangulating different sources of evidence, in order to answer a question. Due to all manner of reasons people do not always answer a question in an accurate or correct manner. Triangulating three or more sources of evidence has been demonstrated in the literature to enhance the accuracy of the inferences reached. In essence corroboration is being pursued between three or more sources of objective evidence.

In practice the three sources of objective evidence that are triangulated are:-

- Interviews

> Observation

> Random representative samples of records

Where the sources of objective evidence triangulate (reconcile), it is induced that the FBO has been doing the right things.

5.0 Combining the Triangulation and the Reality Check (Evidential Triangulation).

References is made to figure Eight below:-

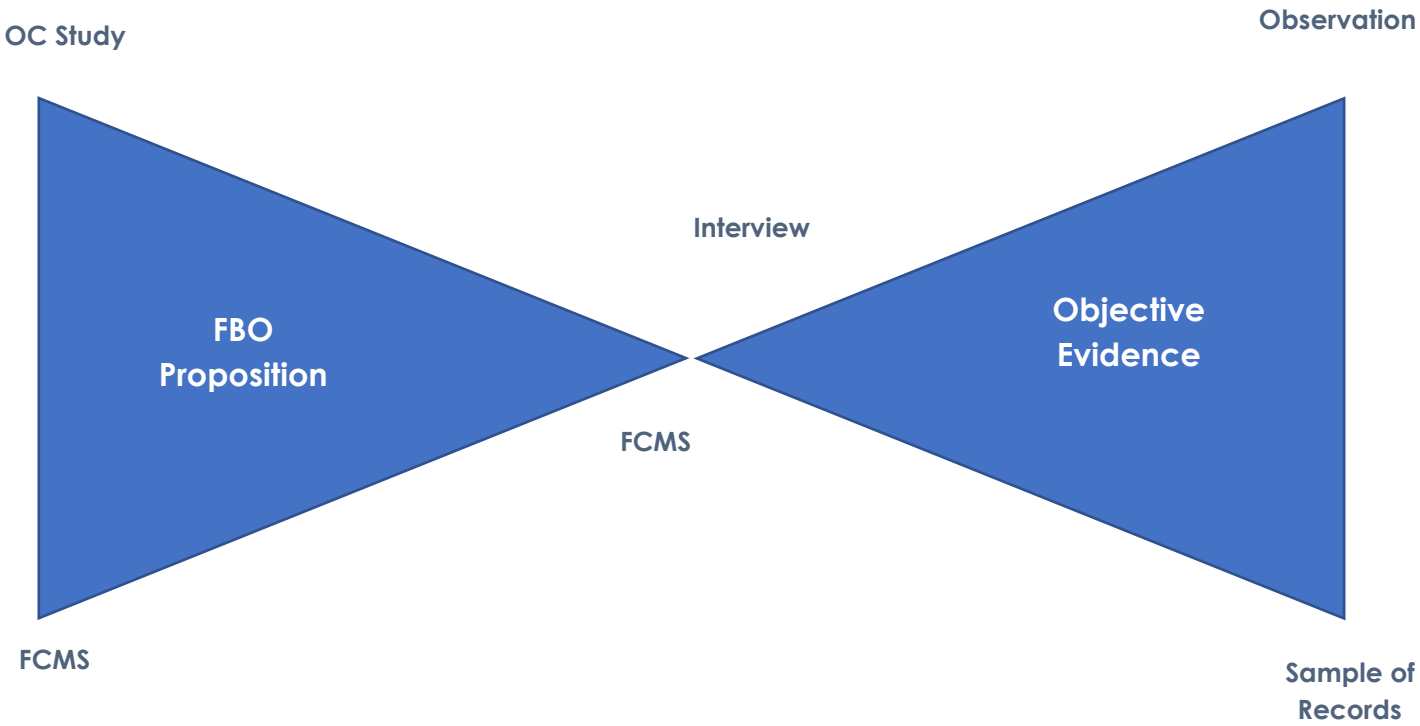


Figure Eight Evidential Triangulation (RHS) and Showing the Relationship with Triangulation (LHS).

This figure represents the relationship between Triangulation and Evidential Triangulation. The LHS i.e. Triangulation addresses the question '**Does the FBO propose to do the right things in the first place?'** and on the RHS i.e. Evidential

Triangulation addresses the question "**Has the FBO actually been doing the things that they have proposed to do?"**

Inspection forms are now being used in this manner which has become known as the '*Bow-Tie'* pattern. Exponents find it much quicker than traditional approaches and inspection forms that pre-prompting and require filling in text fields. They say it gets straight to the point – And clearly guides them to address the salient questions of verification.

One bow tie per proposition is annotated accordingly, i.e. Triangulation on the LHS is annotated with an FBO proposition and the OC Study of that proposition, on the RHS it is annotated with three sources of objective evidence.

5.1 Combining the Triangulation and the Reality Check (Evidential Triangulation) - Food Safety Example Control of *Clostridium botulinum*

Reference is made to Figure Nine below which is a triangulation of the FBO's proposition for the control of *Clostridium botulinum* in vacuum packed cold smoked salmon.

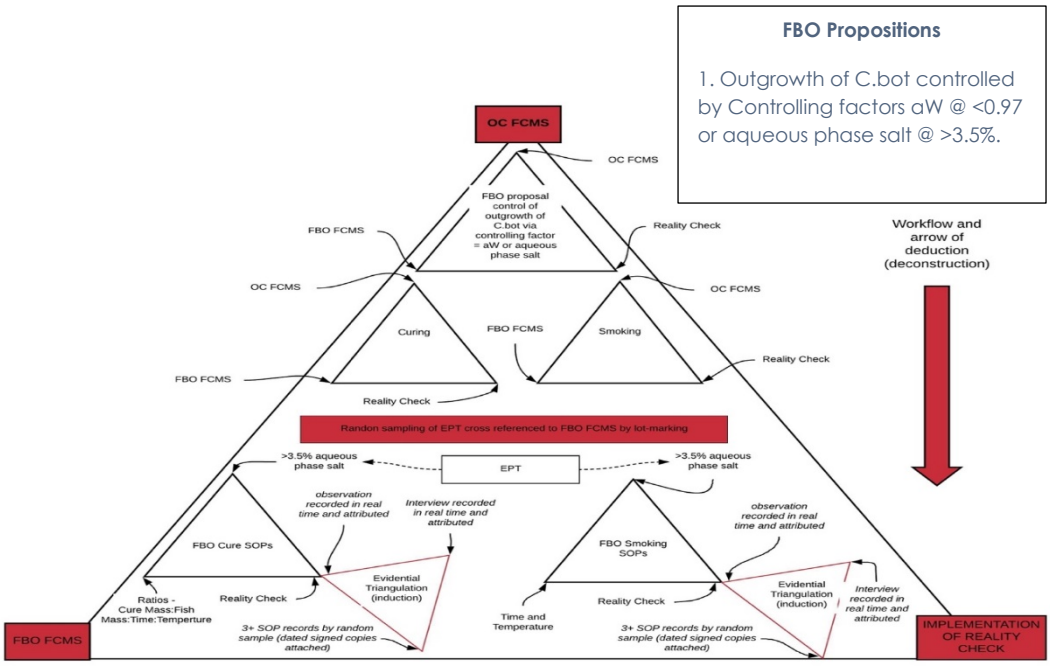


Figure Nine:- Summary Triangulation of the FBO's Proposition for the Control of *Clostridium botulinum* in Vacuum Packaged Cold Smoke Salmon.

The FBO is proposing to control the outgrowth of *Clostridium botulinum* by the application of the Controlling Factors of aW at <0.97 or aqueous phase salt at >3.5%. Following the same approach outlined above, based upon a literature review of the Food Science and Technology of the process and its epidemiology, it is deduced that these Controlling Factors are achieved through a combination of osmotic and drying effects, applied through curing and smoking respectively.

This example demonstrates a different triangulation approach. It should be recalled that OCV is not a process.

It is logical that a passed EPT in terms of aW or aqueous phase salt can be substituted for the OC Study. This because in relation to this proposition those levels of Controlling Factors are exactly what the OC Study would require.

An RRS based EPT result is selected. The Officer then requires the FBO to provide all of the SOPs and their records in relation to the propositions of curing and smoking, using the production code or **Lot Mark** of the final product to trace and reconcile the EPT result with the relevant SOPs and their records. The Officer then proceeds with a Reality Check triangulating three or more sources of Objective Evidence in this case an interview with one of the operatives selected upon an RRS basis for each SOP, and RRS of the production records and RRS based observation of the SOP in action.

If all of this triangulates, then the following can be induced:-

- The FBO has proposed the correct things & has actually been doing those things
- There is an effective FCMS
    - SOPs relate to Controlling Factors and the SOPs are effective in producing the Controlling Factors
    - There is a Traceability and Lot Marking System
    - There is a system of Document Control
    - There is system of record keeping
    - Operatives understand their role and are competent
    - Operatives have been applying the SOPs.

6.0 **Stress Tests**

Triangulation according to the approaches in this primer requires a lot to go right and as such places a significant **stress-test** upon the FCMS. If all aspects

do triangulate, then the inference that the FBO proposes the right things and has been doing them is strengthened.

7.0 **Triangulating FBO's Propositions in Relation to Minimally Processed Foods**

Further repetitions of such an approach, especially where RRS features further increase this confidence.

This is particularly significant where there is lack of C**ause and Effect Transparency**'. This is a feature of many minimally processed, so called '*artisan*' products where '***hurdle-technology'*** is applied. There is no cook-and-kill step and such processes are problematic to validate. Examples are cold-smoked fish, raw- cheese and beef jerky. As long as the FBO does not change anything, then successive iterations of this approach associate the proposed Control Measures with a positive outcome, leading to further inference that the FBO's propositions are sound and true.

It is noteworthy that the timeframe referenced by this approach extends into the past and is cross referenced to the present. Coupled to RRS, this helps address the long-standing criticism that inspections are only a '*snap-shot'* in time which is highly questionable in terms of effective OCs.

8.0 Conclusion

OCV was developed using deductive logic. In its application it uses both deductive and inductive logic and which are mainstays of the scientific method. This basis provides for professional rigour in and recognition of what

we do. OCV has provided the professions with a scientific discipline of their own. From this standpoint the principal aim of effectiveness is achieved, and the secondary objective of consistency is achieved.

Triangulation and Gap Analysis are the fundamental principles of OCV. This provides for a systematic and structured approach embodying scientific metrics of the FBOs performance.

OCV recognises and respects that inspections are in fact complicated activities in their own right - For example the chain of events leading to foodborne illness and to Food Crime can have many and varied permutations. OCV addresses this issue by breaking down and mapping the actual complexity, in order that simplification of it can follow on.

OCV is not a process and different approaches to triangulation have been exemplified.

OCV places exacting '*stress-tests*' on the FCMS. A lot of aspects have to go right for everything to triangulate.

If the FBO's propositions reconcile with the OC-Study and if these propositions can be reconciled with the need to practically implement them, it is deduced that the FBO's propositions that the Food placed upon the market is safe and authentic are true.

Conversely, If the Inspecting Officer is unable to reconcile these aspects, then the deduction is the opposite and the situation is referred to the Enforcement Policy of the relevant Competent Authority.

Section 2: FSS Guide to OCV – HACCP Study Guide

Seafish would like to thank Food Standards Scotland for permitting the inclusion of their guide in this resource pack

Food Standards Scotland
Inbhe Bìdh Alba

sfelc
Scottish Food Enforcement Liaison Committee

OFFICIAL CONTROL VERIFICATION

HACCP STUDY GUIDE

Version 1

Acknowledgment

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Amber Souter - Food Standards Scotland

Introduction

A key component of the approach outlined in the Official Control Verification Guidance (OCV Guidance) is the OCV Study. This is intended to provide an external reference point or standard with which the FCMS is compared and contrasted. The creation of this external reference point is critical to the analysis of the FCMS and it is only through the process of Gap Analysis between these two points that the validity of the said FCMS can be verified. Officers should refer to the OCV Guidance for further detail on this process.

It is acknowledged that the scope of a Food Control Management System (FCMS) is more comprehensive than that of a HACCP and also relates to authenticity and process control etc, however, the scope of this document is specific to the HACCP study which encompasses the management of generic food safety hazards within the pre-requisites programmes and the management of food specific hazards within the HACCP Control Chart

This guidance outlined below represents a methodology for conducting a HACCP study from the standpoint of a food law enforcement officer. This involves the use of algorithms which are intended to represent the officer's thinking, although this should not unduly restrict natural thought processes. The aim is to gauge the adequacy of the FCMS in terms of food safety.

The table below summarises the Steps and the Principles of a HACCP Study.

Verifying Steps 1 to 12 – The steps of the Official Control HACCP study are as follows:

HACCP Step	Task	HACCP Principle
Step 1	Assemble HACCP Team	
Step 2	Describe Product	
Step 3	Identify Intended Use	
Step 4	Construct Flow Diagram	
Step 5	Confirm Flow Diagram	
Step 6	List all potential hazards; Conduct a hazard analysis; Consider control measures	Principle 1
Step 7	Determine CCPs	Principle 2
Step 8	Establish Critical Limits	Principle 3
Step 9	Establish Monitoring	Principle 4
Step 10	Establish Corrective Actions	Principle 5
Step 11	Establish Validation, Verification and Review	Principle 6
Step 12	Establish Documentation and Records	Principle 7

Verifying HACCP Step 1 – Assembling the 'HACCP Team'

Relevant Inspection Stages: Preparation and Opening Meeting

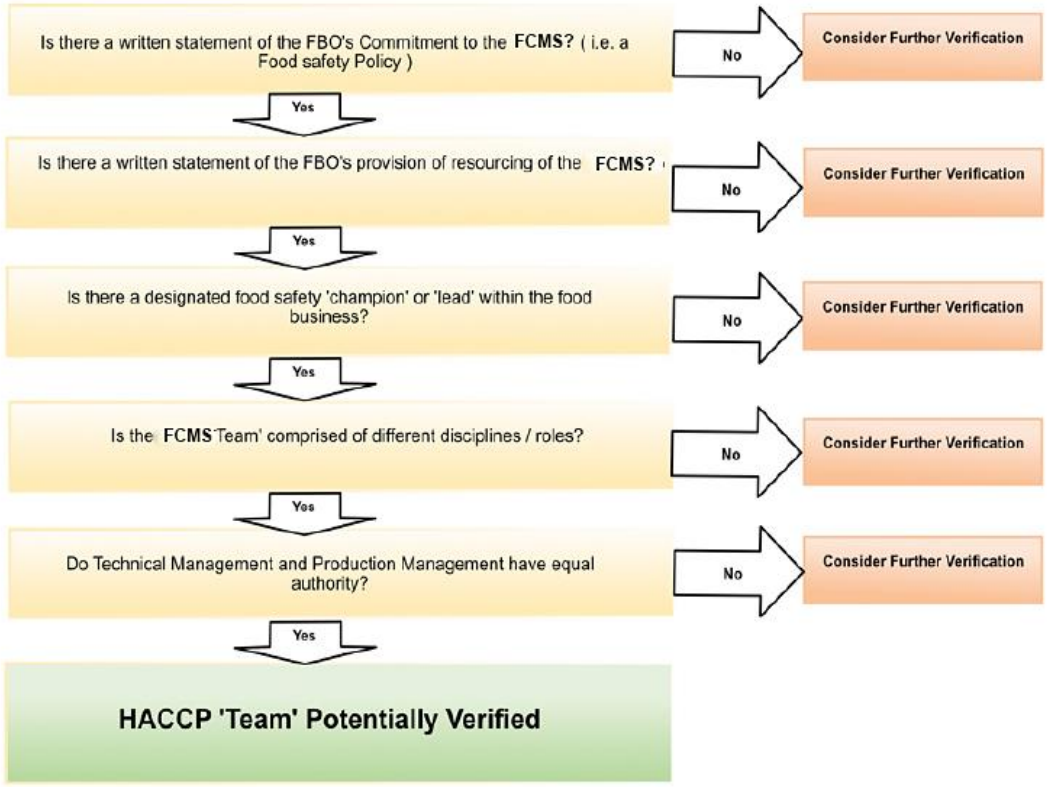
Two key issues at this point are the commitment of the FBO and the competency of the team. Without FBO commitment, the HACCP is significantly undermined. The commitment of the FBO is often considered to be an essential prerequisite in its own right.

The 'HACCP team' must also be competent. Ideally, the team should be multidisciplinary in order to bring specific knowledge and expertise appropriate to the product, the process and the processing environment.

It is acknowledged that the terms 'HACCP team' and 'multidisciplinary' reflect an idealised situation – one that does not often occur in reality, particularly within SMEs. However, these principles remain valid even where there is a degree of scaling applied in terms of the size of the HACCP team in proportion to the size of the business. In effect, competence of the 'HACCP team' is critical, regardless of the scale of the business. (See also OCV Guidance Chapter 5, Form C – FCMS Review Form)

Figure 1 below represents the process, which may be followed when verifying the 'HACCP Team'.

Figure 1: Verification of the HACCP Team



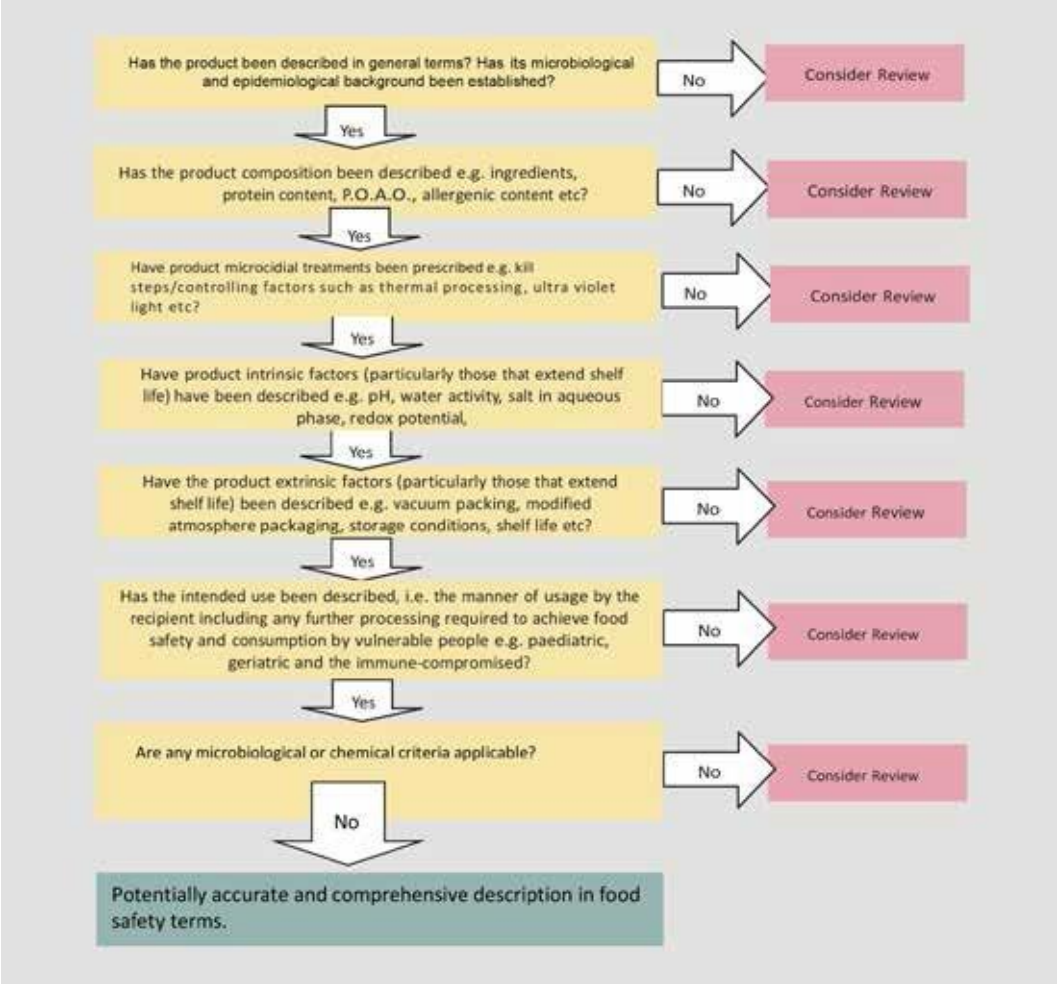
Verifying HACCP Steps 2 & 3 – Description of the products and their intended use

Relevant Inspection Stages: Preparation

The verification of product descriptions is essential as these descriptions represent the foundation of the HACCP. In effect, the design of the HACCP is informed by the product descriptions – and the system must accurately reflect these. The two must be compatible if the HACCP is to be fit for purpose.

Figure 2 below represents the process which may be followed when verifying the description of the product and the definition of intended use. This needs to be supplemented by knowledge of the relevant food hazards and the food science and technology involved. The Officer should conduct research at this stage into the epidemiological history of the product and process. (See also OCV Guidance Chapter 5, Form C – FCMS Review Form).

Figure 2 : Verifying the Product Description



Product descriptions do not need to be extensive or complicated. Concise descriptions need contain only the information relevant to the product in food safety terms as in the following example:

Example A.1 – Product Description

1. Pasteurised Fresh Milk.
2. Composition - Raw bovine milk, origin UK.
3. Rendered safe for human consumption through pasteurisation.
4. Intrinsic Properties – pH 6.5, Aw 0.997
5. Extrinsic Factors – Packaged aseptically in Polybottle with cap
6. Requires chilled storage refrigerate < 5°C, with a shelf life of 14 days and consume within 3 days of opening.
7. RTE suitable for consumption by all.
8. Allergens, Milk.
9. Free from pathogens, <100 cfu/ml. No chemical additives.

Verifying Step 11 – Verification Procedures

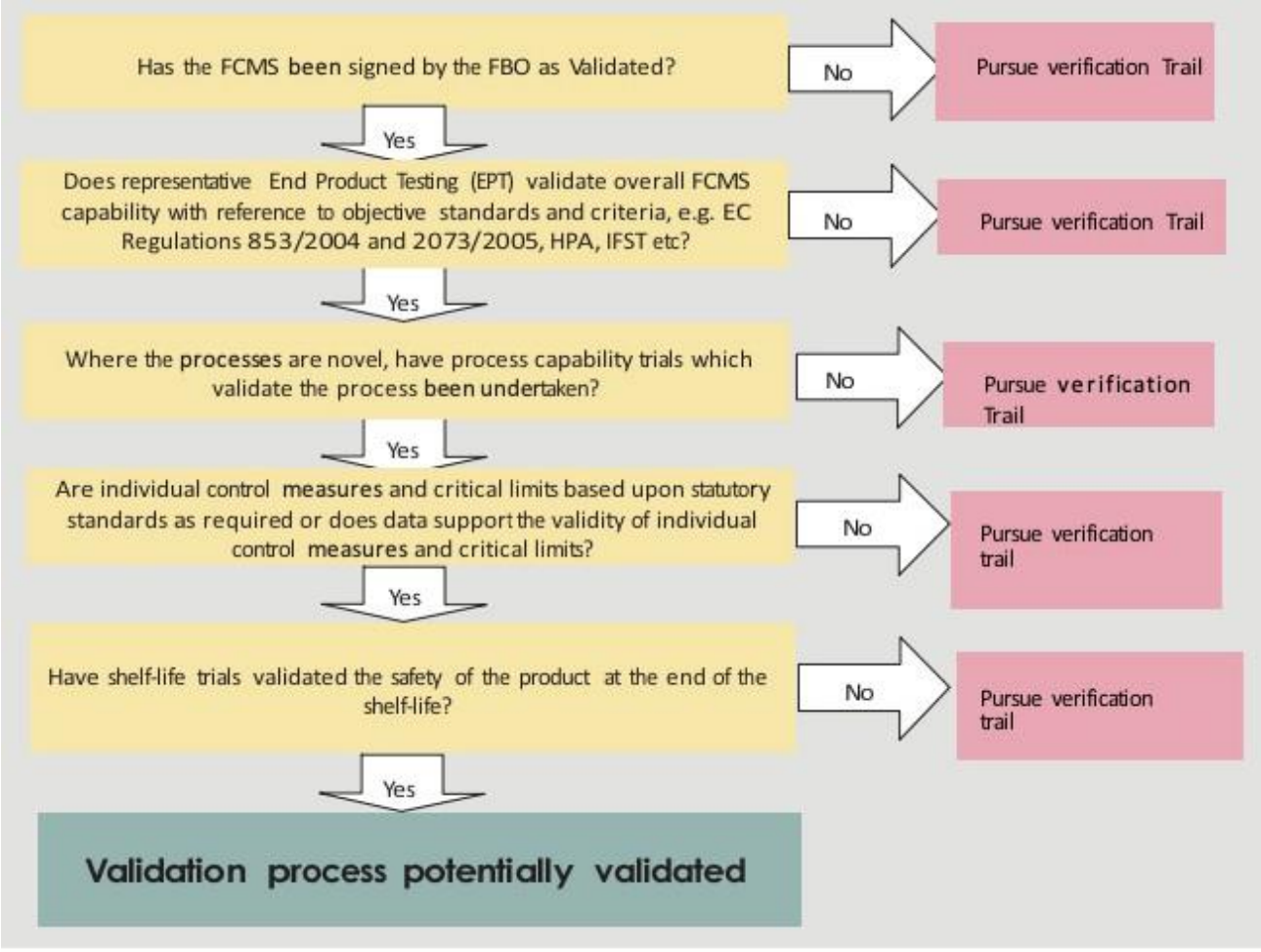
Relevant Inspection Stages: Preparation and Opening Meeting

Verifying Validation

Validation relates to proving the efficacy of the entire FCMS. This must be conducted prior to production (in terms of placing food on the market). Of particular importance is the validation of control measures and of critical limits.

Validation is often, incorrectly, carried out after production has commenced – based upon post-production data. This should not be accepted on safety grounds. The botulism outbreak associated with hazelnut yoghurt described in Example 2.1 of the OCV Guidance illustrates the dangers of inadequate validation. Figure 3 below represents an algorithm which may be used to verify the validity of the FCMS. (See also Chapter 5, Form C – FCMS Review Form).

Figure 3: Verifying Validation



The WHO-Codex document 'Guidelines for the validation of Food Safety Control Measures CAC/GL 69-2008' provides a useful reference.

The Difficulties of Verifying Validation

Verifying validation, particularly in relation to the identification and control of critical points, is a challenging activity and is one area where the officer may require specialist assistance.

Verifying Verification

The verification status of the HACCP itself requires verification during the early stages of the inspection process.

In order to do so, the officer should assess the validation data, end product testing results, internal and external audit documentation as well as the frequency and thoroughness of all verification activities.

The officer should consider whether changes or deficiencies in the HACCP plan, new emerging hazards, etc., are adequately addressed. The officer should also consider what actions are taken as a result of inadequacies in the HACCP (including its prerequisites) or any other non-conformity.

Verifying HACCP Steps 4 and 5 – The Process Flow Diagram

Relevant Inspection Stages: Opening Meeting and Main Inspection

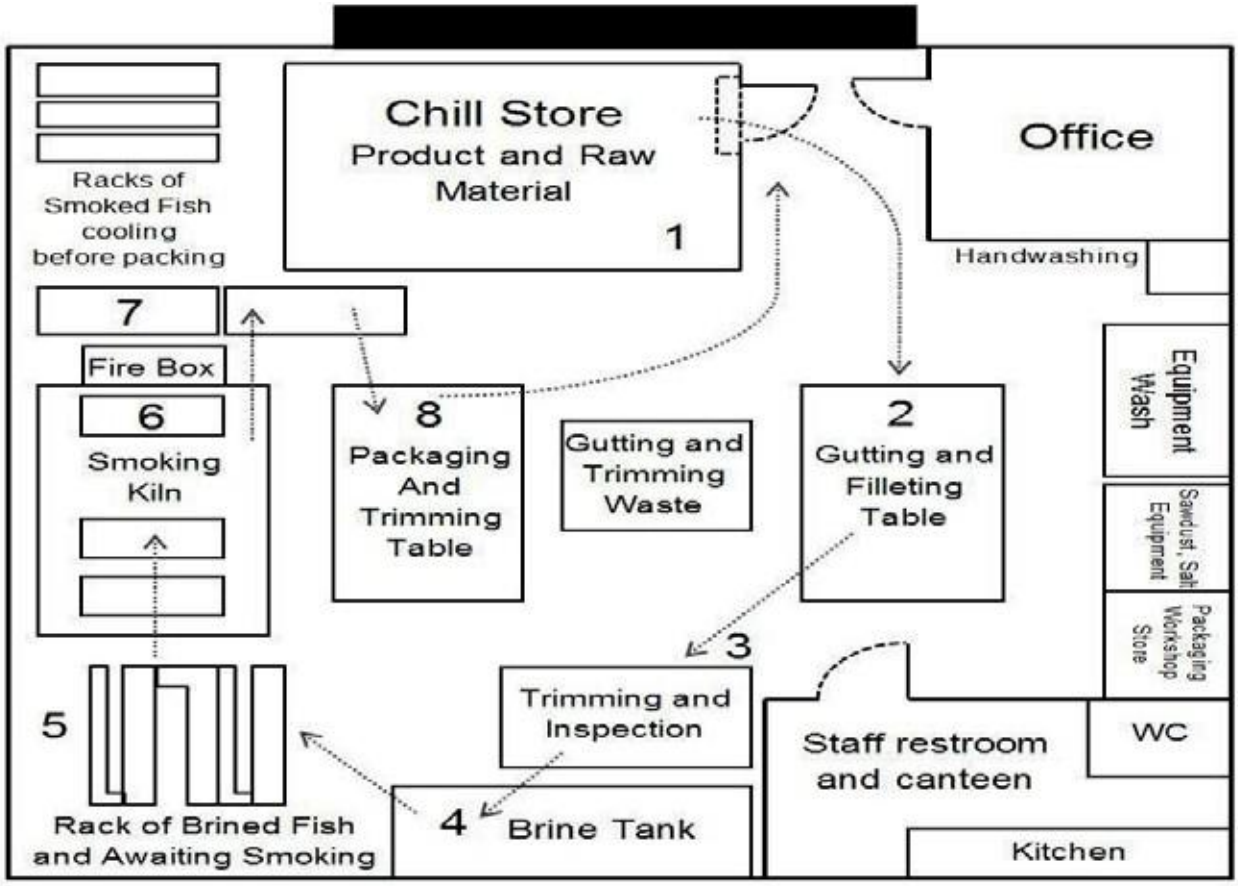
OC Verification of the Process Flow Diagram is vital as deviation from the process flow is frequently the first sign that the actual operation is deviating from the HACCP itself, i.e. the HACCP is becoming invalid. This process will also assist the officer in determining whether the CCPs are correctly identified and also if there is potential for post-process contamination.

Once the validity of the Process Flow Diagram been established, the focus may shift toward verification, i.e. “are they doing what they say they say they are doing?” This involves the same HACCP principles, but with more focus on their practical application.

Verification of the accuracy of the Process Flow Diagram is an essential component of the Official Control HACCP study and of the process of cross- referencing with the FBO HACCP Study. Inaccuracy or error at this stage will have a knock-on effect throughout the subsequent steps of the FBO’s HACCP study, possibly rendering them and the overall FCMS invalid.

Verification is undertaken by a detailed ‘walk of the line’ and careful cross-referencing of the actual operating sequence to the Process Flow Diagram. The officer should systematically and sequentially track the product(s) throughout the entire process flow(s); beginning at the point where raw materials are received and ending at the point where thefinished product is packed or dispatched. Subsidiary processes flows, e.g. staff flows and waste flows may then be subsequently verified. Figure 5 represents the systematic process of tracking the process flows.

Figure 5: Systematic/Sequential Tracking of the Process Flows



The Process Flow Diagram – A Framework for the Remainder of the Official Control FCMS Study

The Process Flow Diagram represents the essential framework for the FBO's HACCP study as well as for the Official Control HACCP (OC HACCP) Study.

The remainder of the OC HACCP Study is undertaken using the verified Process Flow Diagram(s) as its framework. The officer should track the process/packaging/personnel and waste flows applying the following steps of the OC HACCP Study to each step and to the prerequisite programmes.

Verifying Step 6 – List all potential hazards; conduct a hazard analysis; consider control measures

Relevant Inspection Stages: Preparation

Introduction to Step 6 – Listing Potential Hazards, Conducting Hazard Analysis and Considering Control Measures

Step 6 presents perhaps the greatest challenge to the FBO and the officer during an inspection. If the potential hazards and their nature cannot be recognised and understood, the official control process will be compromised.

Despite this, reference to this process in the WHO Codex is quite abstract:

"The HACCP team… … should next conduct a hazard analysis to identify, for the HACCP plan, which hazards are of such a nature that their elimination or reduction to acceptable levels is essential to the production of a safe food.

- *In conducting the hazard analysis, the following should, wherever possible, be included:*
- *The likely occurrence of hazards and the severity of their adverse effects;*
- *The qualitative and/or quantitative evaluation of the presence of hazards;*
- *Survival or multiplication of micro-organisms of concern;*
- *Production or persistence in foods of toxins, chemicals or physical agents; and*
- *Conditions leading to the above.*
- *Consideration should be given to what control measures, if any exist, can be applied to each hazard."*

[WHO Codex 2003]

Step 6 constitutes perhaps the most frequently misunderstood and misapplied element of any HACCP study. A generic approach involving a simplistic requirement to produce process flow diagrams and apply very broad categories of hazards (e.g. biological, chemical and physical) at each step is likely to lead to inadequate controls being applied.

Manufacturing processes are often highly complex, involving multiple product lines as well as numerous production, personnel, packaging and waste process flows. Many of these afford the opportunity for the introduction, multiplication and survival of food hazards. There is also the additional human factor which can lead to pressure to achieve just in time production for high-risk, short shelf-life products.

However, if the underlying science is represented and Step 6 is broken down into bite sized- chunks, the process can be made easier to apply and the outcomes can be more successful.

The approach described here is based upon the science of epidemiology, an approach aligned to OCV and is based on a number of precedents:

- Bryan (WHO Codex ('Epidemiological Contributory Factors concept')) – 1992.
- LACORS (Relevance approach to 'Hazard Mapping') 1993.
- Structured Approach' to Step 6 formulated by Mortimore and Wallace 1998.
- 'Hazard mapping' – Food Law Code Practice Guidance Food Standard Scotland 2014 and antecedents (Note the terms 'Hazard Mapping' is not elaborated upon and must be inferred).

Officers are provided with inspection tools in the form of a Step 6 hazard identification and analysis form (Annexes 1 and 2) and a structured algorithm (Figures 15 and 16) which may be used at any point during the inspection.

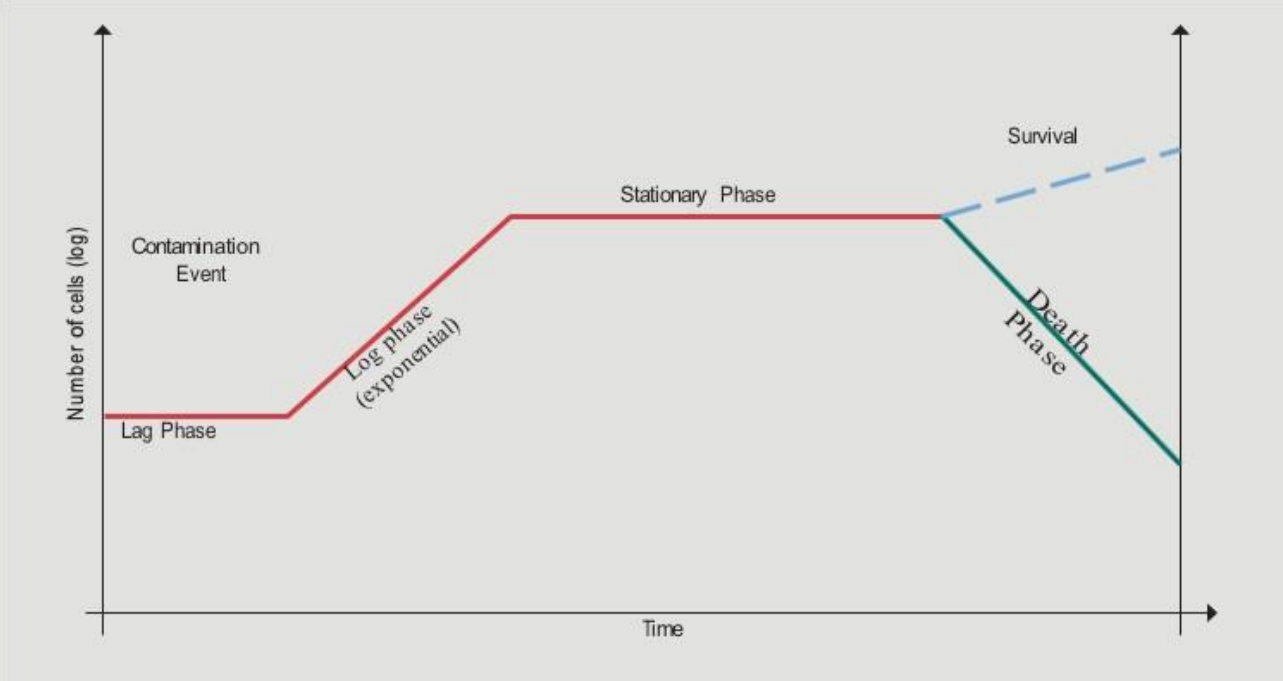
This approach leads to the analysis of the linkages between the process being inspected and the actual causes of food borne disease as confirmed by microbiology and epidemiology.

The Microbiological and Epidemiological Basis of Step

The Microbiological Dimension

Food-borne illness is a predictable natural process that has been described by science. This is represented by the Bacterial Growth Curve in Figure 6 below.

Figure 6: Bacterial Growth Curve



Microbiology confirms that the introduction of a bacterial pathogen by contamination to a suitable growth medium, may exceed an infective dose.

Alternatively, suitable conditions will lead to exponential multiplication of the hazard, eventually exceeding an infective dose, even where the initial contamination level was significantly lower. That said, the hazard may be eliminated or reduced to an acceptable level (i.e. below an infective dose) by the application of conditions designed to achieve that specific aim. Conversely, the hazard may persist if the relevant conditions are not applied. This insight represents the starting point for Step 6.

These relationships can be mapped onto process flow diagrams.

Figure 7 - This diagram represents a key to symbols within the subsequent figures.

	High level of contamination in raw materials	Process cannot reduce hazard to an acceptable level (risk of health adverse effect)
	Incorrect Parameters of Heat Treatment	Legal Limit is exceeded

Introduction of Hazard	Status Quo	Multiplication of Hazard	Elimination of Hazard or Reduction to an acceptable Level

Figure 8 represents the relationship between the process flow and the level of a bacterial hazard relative to an infective dose and a legal limit where the HACCP effectively eliminates the hazard or reduces it to an acceptable level.

Figure 8

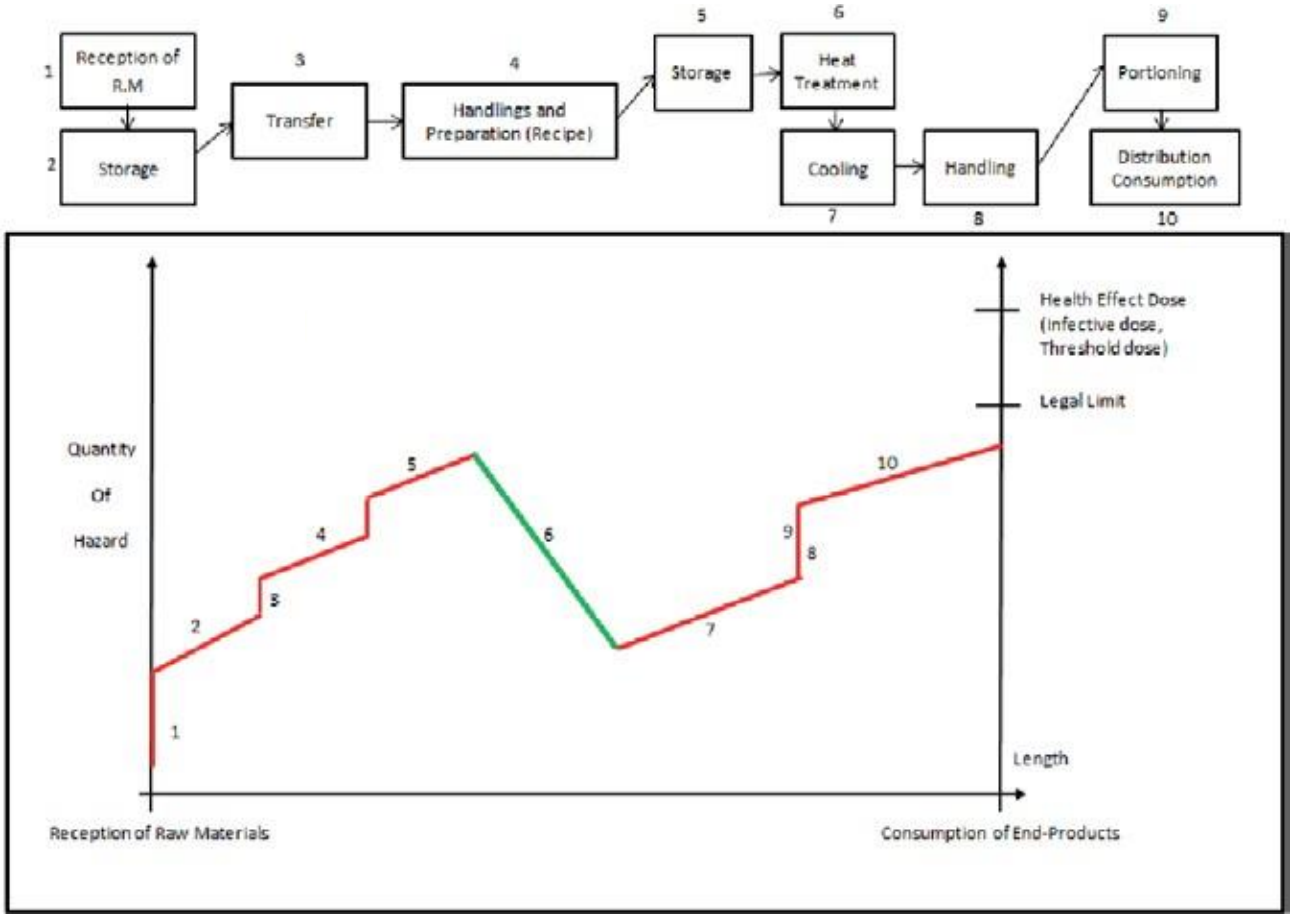
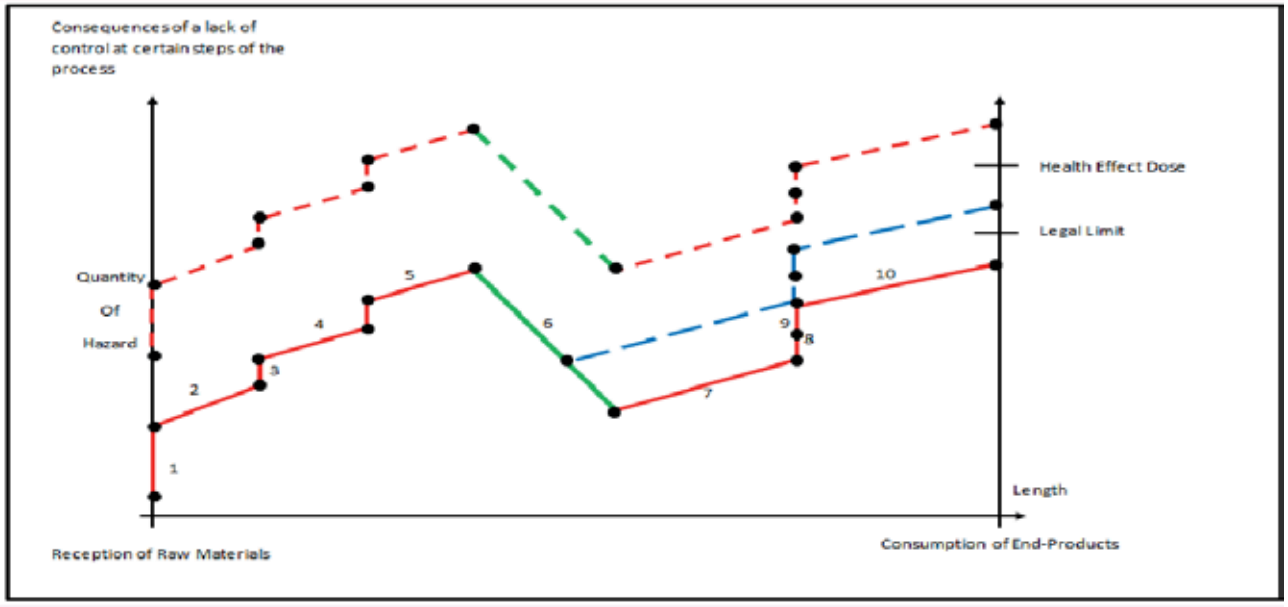


Figure 9 represents a relationship between the process flow and the level of the bacterial hazard relative to the infective dose and a legal limit in a situation where the HACCP has failed to eliminate the hazard or reduce it to an acceptable level.

Figure 9



This is, in fact, what happens in an outbreak of foodborne disease.

The Epidemiological Dimension

Epidemiological data on those factors that are known to have contributed to outbreaks of foodborne disease or practices or situations that have led to outbreaks have been termed as **'contributory factors'**. These 'contributory factors' have been found to be remarkably similar over a range of incidents. Summarised here are the most common 'contributory factors' of outbreaks of food borne disease:

Presence of hazards as inherent contaminants of foodstuffs at the outset of a process;

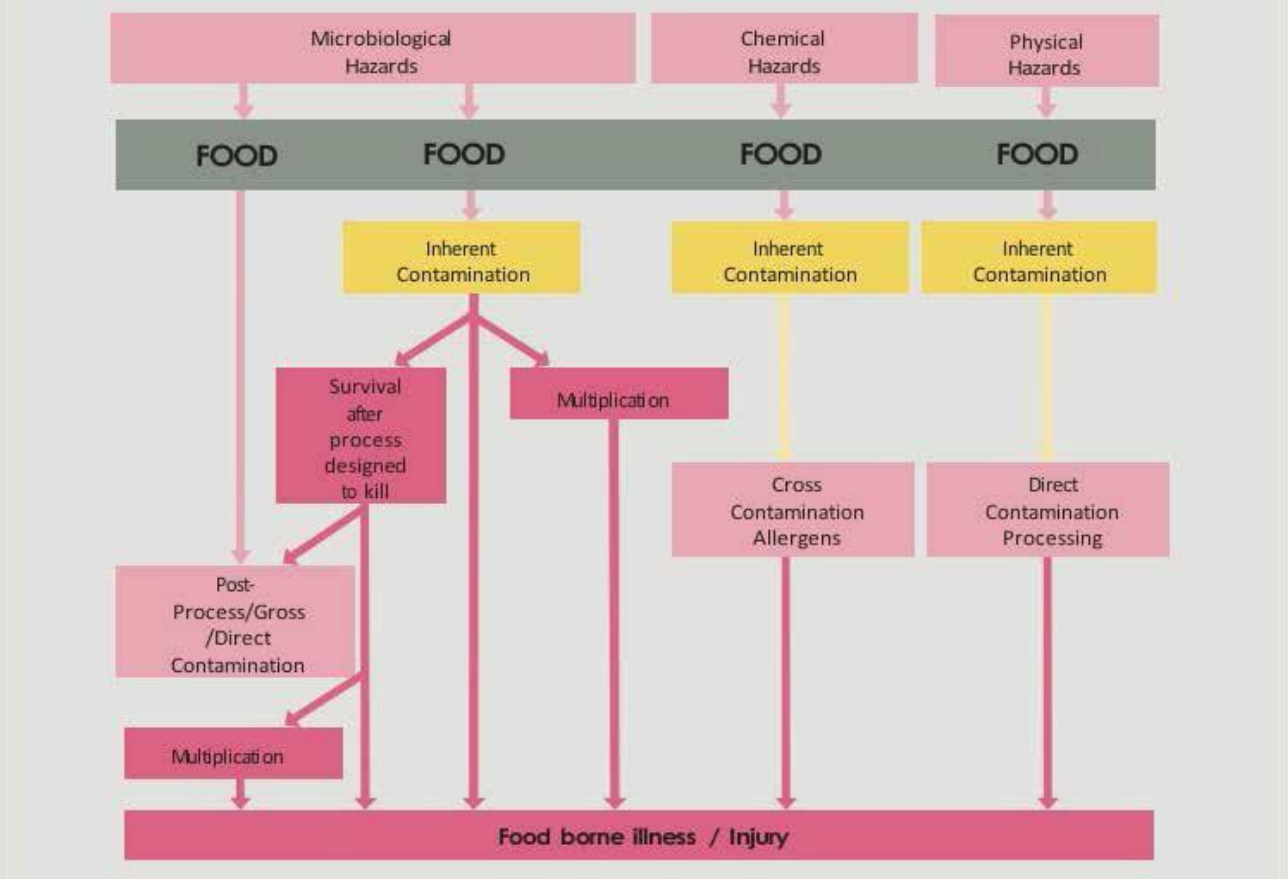
Introduction of hazards by direct contamination or by cross contamination;

Multiplication of hazards; and

Survival of hazards of a process intended to eliminate the hazard or reduce it to an acceptable level.

Epidemiology has confirmed that food-borne illness is the culmination of a predictable 'chain of events' wherein the contributory factors act alongside the hazards to cause foodborne illness. Figure 10 below summarises such a chain of events leading toward food-borne illness.

Figure 10: Food Borne Illness as the Culmination of a Chain of Key Events



The Outcomes – An Officer's Tool for Step 6

These insights lead to outcomes that make a critical contribution to the practice of Official Control HACCP Study:

1. The contributory factors and the chain of events, as demonstrated, reduce the inherent complexity of a manufacturing process and render it more amenable to understanding, thus easing the Step 6 of an Official Control HACCP Study.

2. Considering the list of hazards in terms of the contributory factors concept provides the officer with a practical tool whereby they can **'map'** the hazard onto the process flow diagram where it is relevant to do so. This creates a direct link between the hazard and the process flow diagram in terms of microbiology and epidemiology. The Process Flow diagram and the Hazard Map actually becomes descriptive and predictive of the chain of events that would occur if the FBO did not apply control measures.

3. The key steps within the chain of events renders the chain amenable to interventions (Control Measures on behalf of the FBO and Enforcement on behalf of Official Controls) which prevents the chain of events unfolding i.e. prevent food borne illness, thus being founded on epidemiology – Official Control verification is effective as an Official Control as is required by Regulation (EC) 882/2004.

This practical approach is further elaborated upon below.

A Structured Approach to Step 6

Addressing the problem of Step 6 can be eased by reducing the issue to basic questions, i.e. what, where, when and how etc (see Annex 2). Issues of significance and relevance can then be assessed using the process flow diagram as a framework or map. This approach breaks down step 6 into the following inspection skills:

- Hazard identification, i.e. **what** are the hazards?
- Hazard analysis, i.e. **what** are the significant hazards?
- Hazard mapping, i.e. **when** and **where** the hazards are relevant at process steps?
- Hazard causation, i.e. **why** the hazards are relevant at a process step?
- Hazard control, i.e. **what** needs to be done to eliminate the hazard or reduce it to an acceptable level?

This approach is founded in the microbiology and the epidemiology represented above. The answer to each question is a consequence of the answer to the preceding question. This logical approach ensures that the correct hazard is identified, analysed, mapped and its causation understood. Similarly, the outcome is a logically derived Control Measure that will actually eliminate the hazard or reduce it to an acceptable level.

Hazard Identification (What are the hazards?)

This aspect is relatively straightforward. A gap analysis is undertaken between the FBO's own hazard identification and validated sources of information which define and describe the hazards associated with the same or similar products and processes. Examples of such sources include:

1 Published epidemiological data;

2 Food microbiological textbooks; and

3 Advice from relevant specialist sources.

Officers should be aware of the problems associated with generic groupings of hazards such as 'microbiological, chemical and physical' etc. Bacteria, for example, have different physiological growth requirements which in turn mean that they are opportunistic contaminants under differing conditions. Consequently, species-specific control measures are sometimes required to control them. Officers should be prepared to undertake the appropriate research. Control measures for each hazard specific to more than one contributory factor are frequently required at the same process step.

Allergens and additives are considered as types of chemical hazards.

Hazard Analysis (What are the Significant Hazards?)

The WHO Codex defined hazard analysis as follows:

Hazard analysis: The process of collecting and evaluating information on hazards and conditions leading to their presence to decide which are significant for food safety and therefore should be addressed in the HACCP plan.

[WHO Codex 2003]

These considerations relate to risk factors associated with the identified hazards – the purpose being to identify the significant hazards. In practice, considerations will always include a combination of the following:

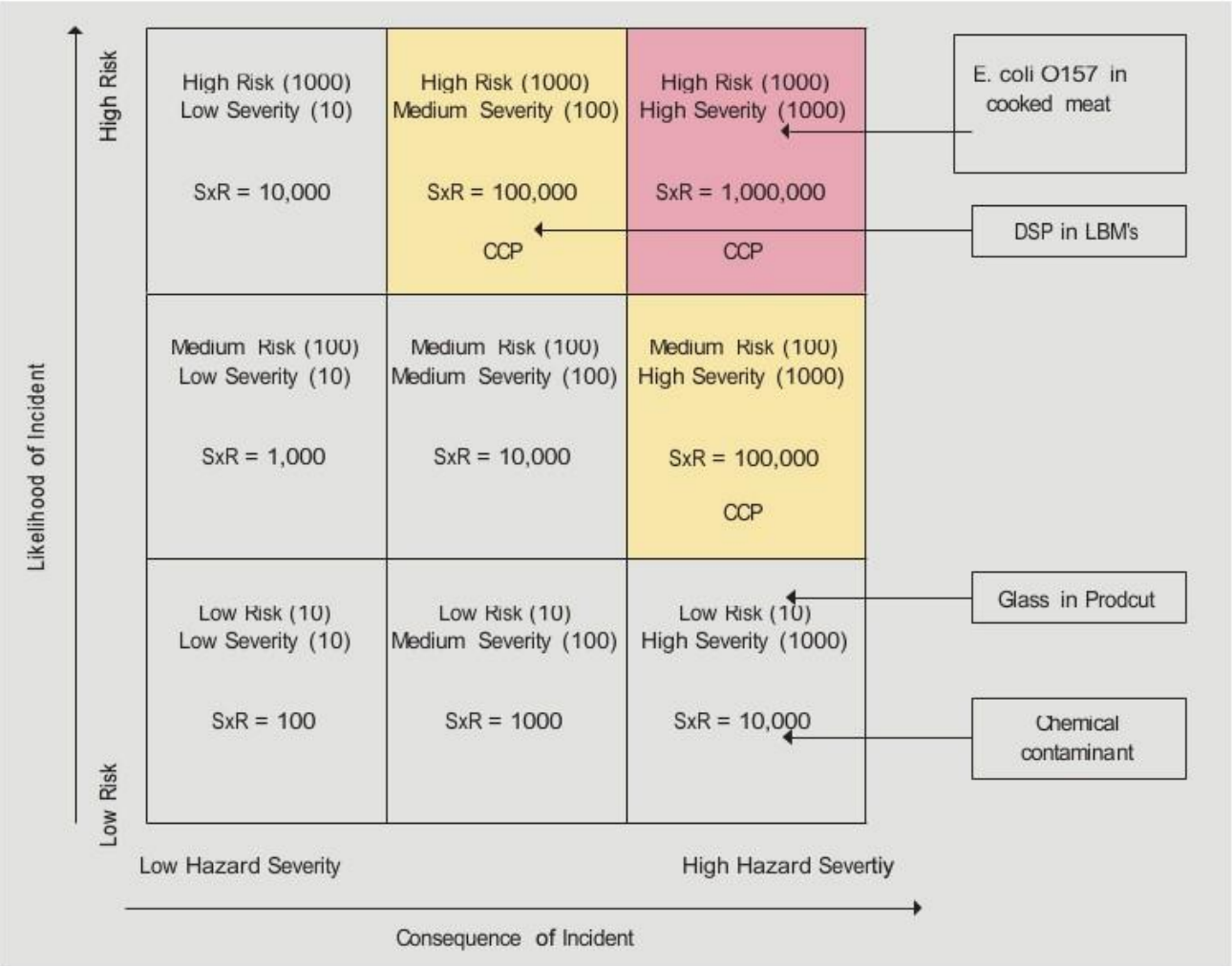
- The likelihood of the hazard occurring and its consequent effects – e.g. previous
- company/industry experience or complaints, epidemiological data;
- The severity of the hazard – e.g. life-threatening/ mild; chronic/acute;
- Numbers potentially exposed to the hazard –
- e.g. lot size; distribution;
- Age/vulnerability of those exposed – e.g. young/elderly; allergies;
- Survival or multiplication of micro-organisms of concern;
- Production or persistence in foods of toxins, chemicals or physical agents; and
- Source or cause of the hazard or conditions leading to the above.

The Practical Problems of FBO Risk Assessment

Since the WHO-Codex guidelines on HACCP were revised and with the advent of BRC and ISO2203 accredited FCMSs, it has become common place to introduce a measure of quantification (i.e. Risk Assessment) to step 6.

Figure 11 below, which summarises this process, is presented in the form of a 'Risk Quadrant' which is a simple formulation of the Risk Assessment Process, where risk is plotted as some function of the likelihood of an incident and the consequences of an incident.

Figure 11: The Risk Quadrant



This process should be approached with caution as errors at this step can lead to significant hazards not being subject to HACCP Principle 2, i.e.Critical Control Point determination. A common error is for such Risk Assessments to be undertaken by unqualified personnel and/or on the basis of incomplete information.

Where officers come across this approach, they are advised to verify the process in sufficient detail to determine whether or not significant hazards have in fact been discarded from HACCP Step 7, i.e. Determination of Critical Control points.

Hazard Mapping (Where and When are the Hazards Relevant at Process Steps?)

The relevance of a hazard to a step in the process flow or prerequisite programme is identified by identifying the relevant contributory factor:

Presence – A hazard which is likely to be an inherent contaminant of the food at the outset.

Introduction – A hazard introduced by contamination at a particular step of the operation, either via direct or indirect cross contamination.

Multiplication – A hazard may increase, e.g. by microbiological growth or toxin production, at a particular step.

Survival – A hazard might survive a particular step designed to eliminate it or reduce it to an acceptable level.

N.B. This is applicable to both generic hazards (prerequisite programmes) and to food-specific hazards (HACCP Control Chart).

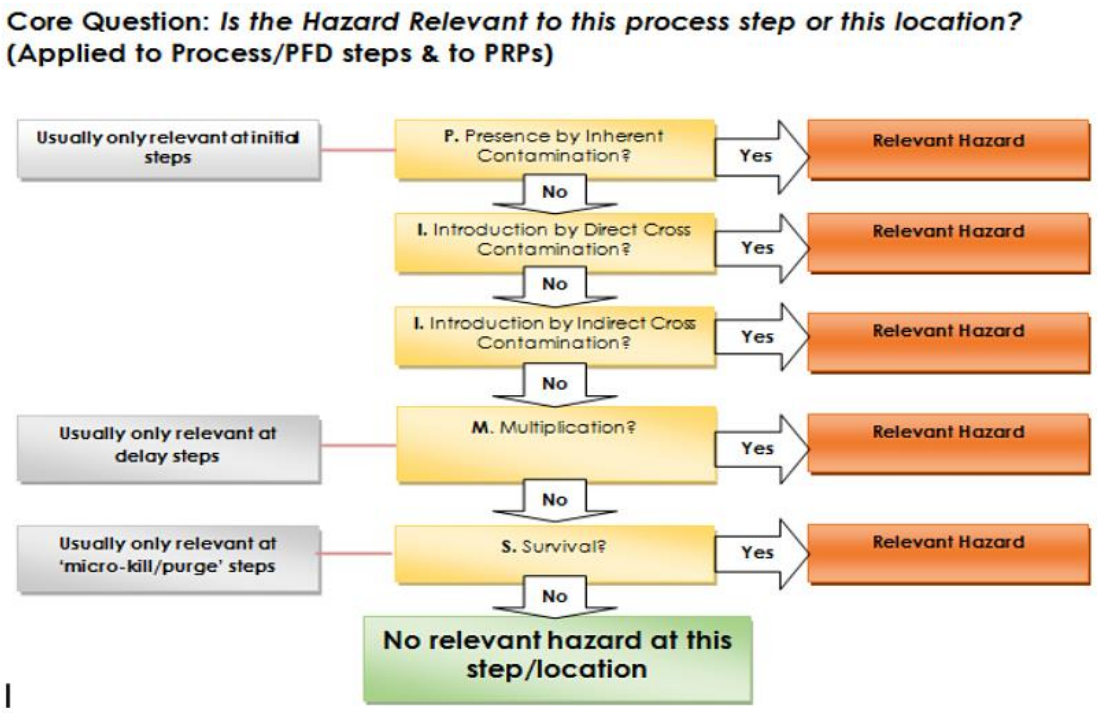
These 'contributory factors' have been condensed into the mnemonic of P.I.I.M.S.

These 'contributory factors' can also be integrated into a structured algorithm. This practical tool for officers can be applied to the process/personnel and waste flows and/or prerequisite programme, in order to identify whether the hazards are relevant at the process step or prerequisite in question.

The algorithm in Figure 12 below may be used to verify the relevance of a hazard at any step in the process flow(s).

Figure 12 Algorithm for Considering the Relevance of Hazards at Process Steps and Prerequisite Programmes

Core Question: Is the Hazard Relevant to this process step or this location?
(Applied to prerequisites or the process flow)



The outcome of this process is the Hazard Map which identifies the hazards in terms of the contributory factor at each process step.

Figure 13 demonstrates the process of hazard mapping.

Figure 13: Hazard Mapping (Microbiological)

Hazard Mapping (Microbiological)	
Manufacturing	Fish Products Smokery
Delivery (a)(b)(c) | Storage (b)(c) | Preparation (b)(c) | Processing (b)(c)(d) | Post Process (b)(c) | Storage (b)(c) | Distribution (b)(c) | Customer (b)(c)(d)	Delivery (a)(b)(c) | Storage (b)(c) | Fillet and De-bone(b) | Brine/Cure(c)(d) | Smoke (d) | Chill (c) | Vac Pack (b)(c) | Store (c) | Distribution (b)(c) | Customer (b)(c)(d)

Key: (a) Present (b) Introduction (c) Multiplication (d) Survival

Using hazard mapping the officer is able to verify exactly where the significant hazards are relevant within an establishment in terms of a potential chain of events leading to food borne illness.

Hazard Causation (How relevant are hazards at a process step?)

It is recommended that hazard causation is conducted as a relatively straightforward process of deduction, where the starting point is the hazard mapping of the position of the hazard in the process flow diagram and the relevant contributory factor used as the basis for deducing the causation. It is recommended that this is confirmed during the inspection reality check.

Hazard Control Measures. (What needs to be done to eliminate the hazard or reduce it to an acceptable level?)

Control measures must eliminate the identified significant hazards or reduce them to acceptable levels. Processes that do not achieve this objective are invalid as control measures.

Verification is a deductive process and an extension of the HACCP Step 6 process outlined above. By considering the relevant and significant hazards together with their Contributory Factors in the context of causation, the control measure can be logically and accurately deduced. Figure 14 below exemplifies this process.

Figure 14: Contributory Factors and Controls

Contributory Factors	Causation	Control Measure
Inherent Contamination	Contamination at Source	Elimination or reduction to acceptable Levels at Source
Direct or Cross Contamination	Process whereby hazard is transferred directly or indirectly from a contaminated sources to a RTE product	Spatial or temporal Separation of source or vehicle and RTE product and/or cleaning and disinfection
Survival	Failure of a process step to kill the hazard	Process step that kills the hazard
Multiplication	Time and/or temperature promoting multiplication	Time and/or Temperature arresting multiplication

A structured Form for Step 6.

The template Form that officers may use to verify an FBO's own approach to step 6 during an official control HACCP study is included at Annex 1 (See also Chapter 5, Form C – FCMS Review Form)

Chemical and Physical Hazards

The above approach is also used to address Step 6 in terms of chemical and physical hazards. Such categories of hazards are also addressed according to the same process, i.e.

1 Hazard identification, i.e. what are the hazards?

2 Hazard analysis, i.e. what are the significant hazards?

3 Hazard mapping, i.e. when and where the hazards are relevant at process steps?

4 Hazard causation, i.e. why the hazards are relevant at a process step?

5 Hazard control, i.e. what needs to be done to eliminate the hazard or reduce it to an acceptable level?

It should be noted that both chemical and physical hazards must contaminate products for there to be a food safety issue. With physical hazards, there often is an inherent contamination. Typical examples of chemical cross contamination include allergenic residues on machinery and biotoxin accumulation where shellfish are transferred into contaminated waters. These hazards can survive processes intended to eliminate them or reduce them to acceptable levels. Examples include the sieving of flour to remove stones and the separation of product lines containing allergens. Such hazards can actually also multiply, e.g. a metallic foreign object being splintered into multiple shards within a reversing dough breaker, or marine biotoxins increasing where shellfish are conditioned while exposed to direct sunlight.

Verifying Step 6 – Addressing Hazards via Prerequisite Programmes or CCPs

Relevant Inspection Stages: Preparation for inspection

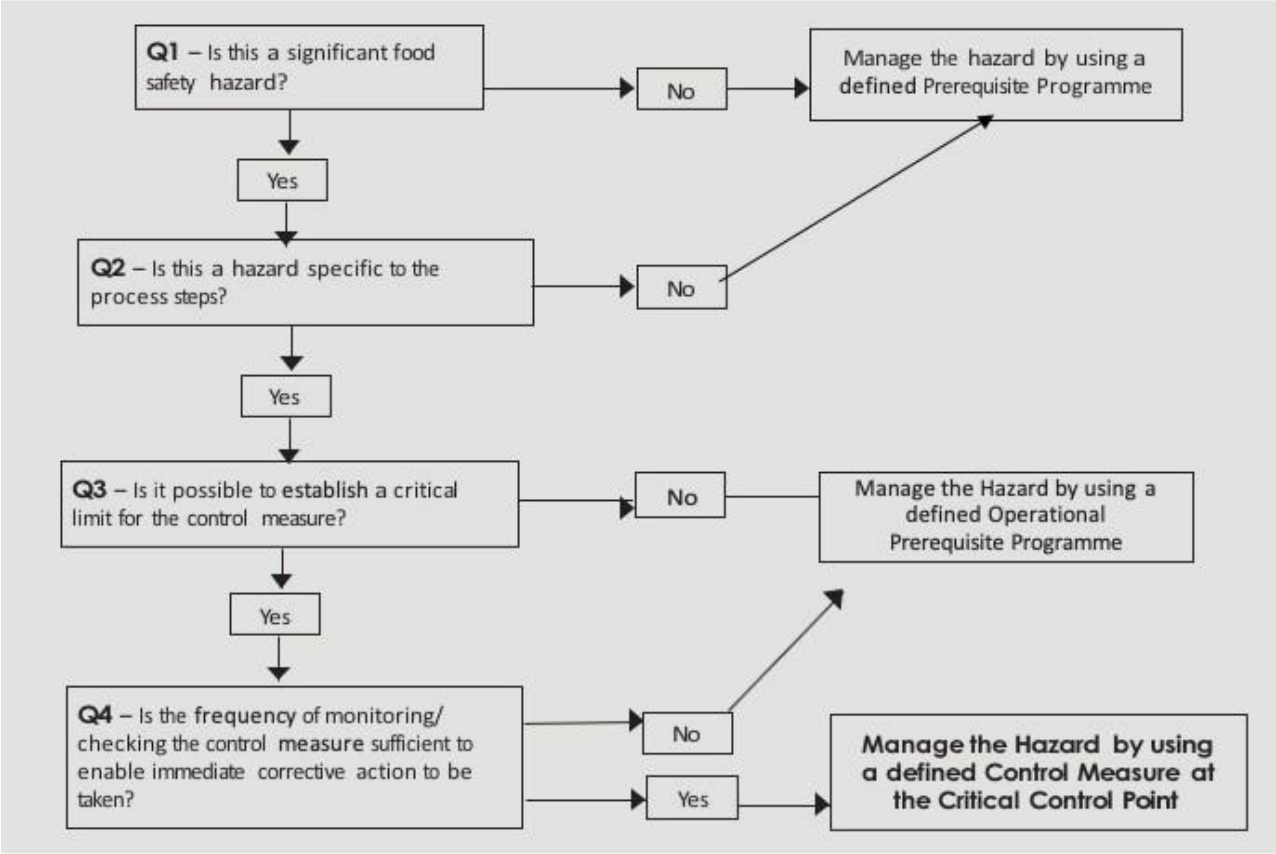
With the advent of ISO 22003, the concept of operational prerequisite programs (OPPs) is now commonplace. In some food manufacturing establishments, OPPs have completely replaced CCPs. One consequence is the need to depart from the established structure of a WHO Codex- based HACCP study which progresses from step 6 to step 7, by the insertion of a new step relating to OPPs before progressing onto step 7. At the time of writing there is no settled, industry-wide conception of OPPs. However, OPPs are intended to embody the control measures for significant hazards that are not amenable to control at specific points in space and time, i.e. at critical control points. Such hazards may be mapped onto the process flow diagram as being relevant at a number of process steps, i.e. they may be "site wide" hazards. It has also become appropriate to consider whether the hazards at the various process steps can be amenable to on- line continuous monitoring (i.e. in real time) and by reference to a discrete, i.e. numeric type critical limit.

Figure 15 below is suggested as a practical tool for officers to verify an FBO's decision to address a hazard as being controlled by a PrP an OPP or via a CCP.

Questions 1 and 2 focus on hazards at each process step.

Questions 3 and 4 focus on control measures.

Figure 15 Decision Tree



Verifying HACCP Step 7 – Verification of the Determination of CCPs

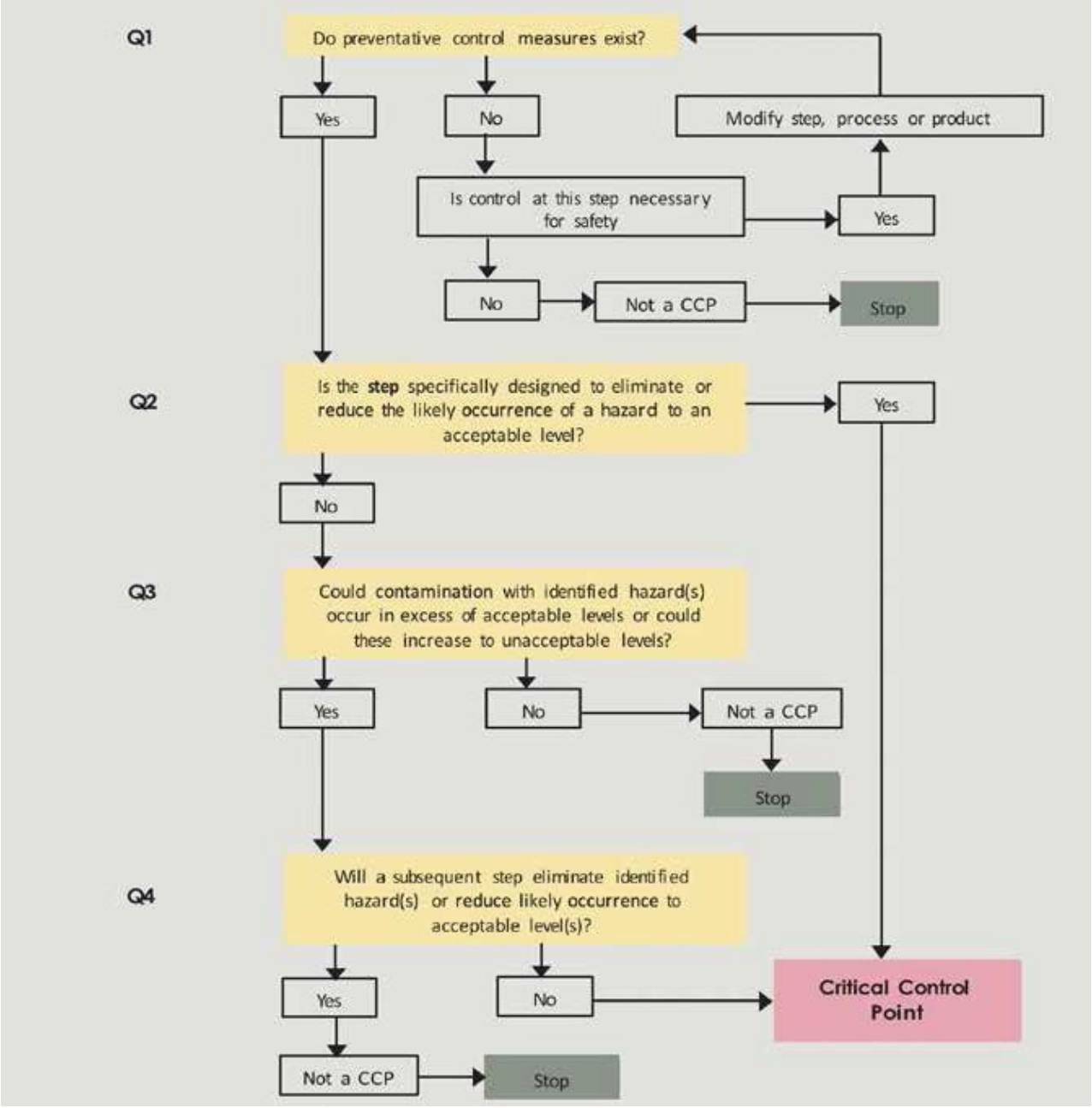
Relevant Inspection Stages: Preparation for Inspection

The verification of the determination of Critical Control Points is critical to the verification of a HACCP. Figure 16 reproduces The WHO-Codex Decision Tree, which is used for the purposes of this verification. The officer applies each significant hazard to every step within the product process flow to the decision tree.

One common error is the omission of Q3. Q3 performs a vital function i.e. the determination of whether the hazard is present at unacceptable levels or may increase to unacceptable levels.

In considering the increase in the hazard, the processing environment should be taken into account (e.g. personnel and equipment which represent a source of contamination).

Figure 16 Decision Tree for Verifying the Determination of CCPs

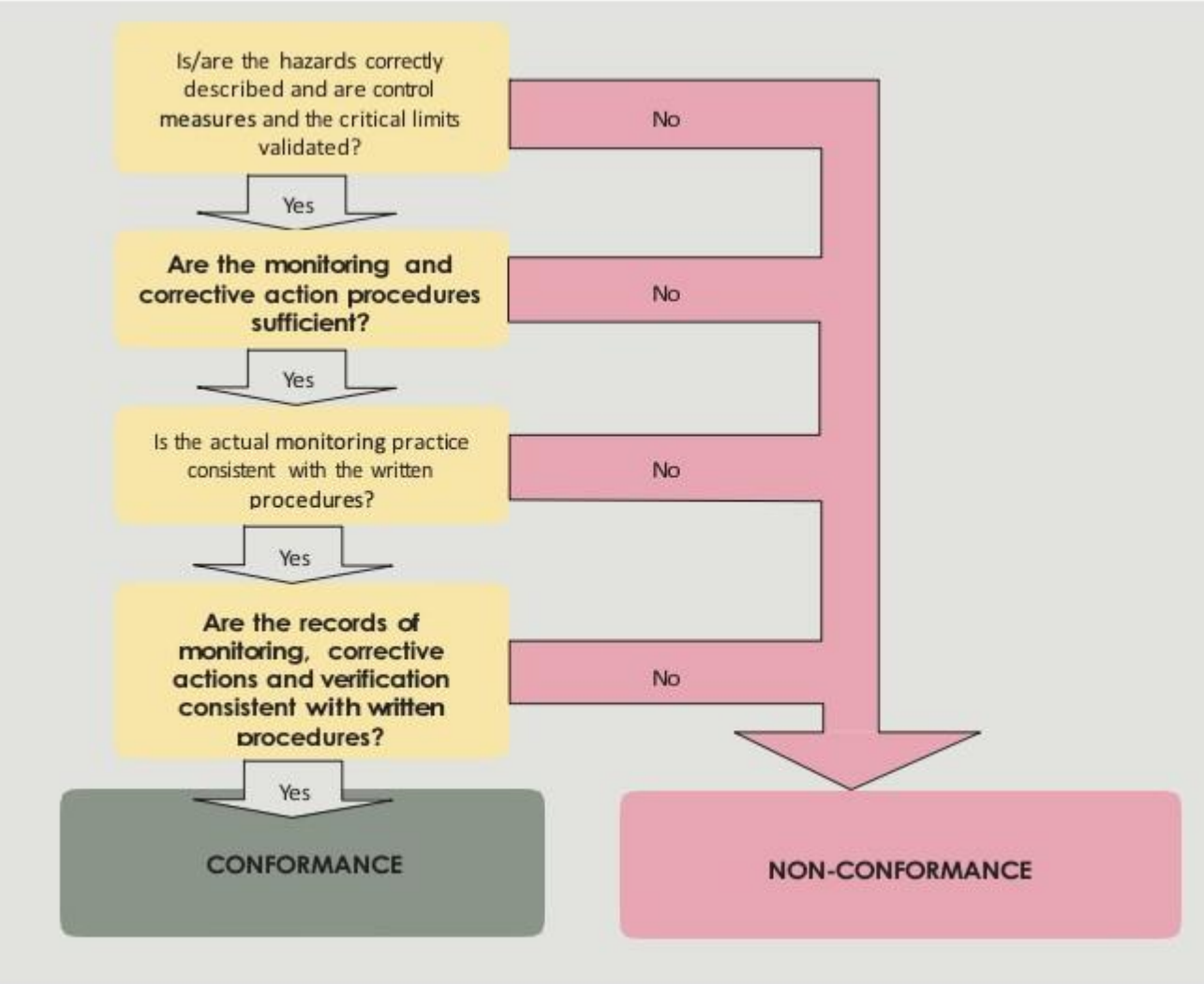


Verifying HACCP Steps 8 to 12 – Critical Limits, Monitoring Systems, Corrective Actions and Record Keeping

Relevant Inspection Stages: Preparation for Inspection and Main Inspection

HACCP Steps 8 to 10 and 12, relate to activities performed at CCPs. Verification may be performed as a straightforward process of compliance auditing, using the algorithm in Figure 17 below.

Figure 17 HACCP Steps 8 to 10 and 12



Process Step	Hazard Identification	Epidemiological Descriptor (P.I.I.M.S)	Causation and/or Source	Hazard Significance Assessment	Control Measure

*[1] 5 W 1 H

Structured & Epidemiological 5 W 1 H *[1] Approach to Step 6/Principle One:- Hazard Identification & Analysis Chart

= Inductive

= Deductive

1. What has been the epidemiological history of this product/process?_____

_____

_____

3. What (if any) are the contaminants/hazards?_____

_____

Process Step Number & Description (Where & When?)	Identification/ List Classify (What?)	*[2] Significance (Risk) (What?) Refer to Risk Quadrant etc LxS			Epidemiological Relevance Descriptor 3* (P.I.M.M.S) (Contributory Factor/ Manifestation of Hazard Qualitative Approach) (How?) *4	Causation/Source (Why?)	Control Measure (What we need to do to eliminate or reduce the hazard to an acceptable level?)

*[2] Risk Quadrant

*3 Mnemonic - P.I.M.M.S/P.I.M.S/P.I.G.S

P =Presence by inherent contamination
I= Introduction by direct contamination
I= Introduction by cross contamination
M= Multiplication
S = Survival

*4 P.I.M.M.S Algorithm

Section 3: Appendices

Bryan F 1978 Factors contributing to outbreaks of foodborne Disease USA

Roberts et al 1982 Factors contributing to outbreaks of food poisoning in England

Journal of Food Protection Vol. 41, No. 10, Pages 816-827 (October, 1978)

Factors that Contribute to Outbreaks of Foodborne Disease

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ABSTRACT

Factors that contributed to foodborne outbreaks that were reported in the U.S. from 1973-1976 are identified and categorized by disease and by locale in which incriminated foods were mishandled. Data from the same years are tallied separately and combined with data from the years 1961-1972. Inadequate cooling was associated with most foodborne outbreaks, with many bacterial foodborne diseases (such as salmonellosis, staphylococcal food poisoning, and *Clostridium perfringens* gastroenteritis), and frequently with outbreaks that originated from foods prepared in foodservice establishments and homes. Inadequate cooling practices were usually either failure to refrigerate cooked foods or the storing of foods in large stock pots or other large containers that were refrigerated. Other important factors that contributed to foodborne outbreaks were the lapse of a day or more between preparing and serving (coupled with inadequate refrigeration or hot-holding during this time), handling of cooked foods by infected persons, inadequate cooking or other thermal processing, inadequately high temperatures during hot storage, inadequately high reheating temperatures, inadequate cleaning of kitchen or processing equipment, ingestion of contaminated raw food or ingredient, and cross contamination. The frequency of involvements of the factors that contributed to outbreaks in England and Wales was quite similar. The principal factors that contributed to staphylococcal food poisoning were inadequate cooling practices, infected person manipulating cooked food, and lapse of a day or more between preparing and serving. The principal factors that contributed to *C. perfringens* gastroenteritis were inadequate cooling practices, lapse of a day or more between preparing and serving, and inadequately high temperatures during hot-holding and reheating. The principal factors that contributed to salmonellosis were inadequate cooling practices, contaminated raw ingredients, inadequate cooking or thermal processing, and cross-contamination.

Epidemiologic and research data collected over the past century have demonstrated that the following sequence of events must occur for persons to get a foodborne disease: (*a*) the etiologic agent must be present either in citizens of a community, in food-source animals, or in the environment in which foods are grown, harvested, processed, or stored; (*b*) the agent itself or the organism that produces it (if it is one of several toxins) must contaminate a food during the growing period or during harvesting, processing, storage, or preparation; (*c*) then, one of the following events must happen; (*i*) the agents must be present on or in the contaminated food in sufficient numbers or concentrations to survive the remainder of the growing period, storage, and processing and still cause illness; (*ii*) bacteria on or in foods in insufficient numbers to cause illness must multiply and reach quantities or produce toxins in sufficient quantities to cause illness; (*iii*) microorganisms, particularly bacteria, enter food preparation areas on or in raw foods, where they are transferred to worker's hands or to equipment surfaces, which if inadequately washed will then contaminate other foods that they subequently touch (and hence, if bacteria, multiply as described in (*3ii*); (*d*) sufficient quantities of the contaminated food that contains enough of the agent to exceed a person's resistance-susceptibility threshold must be ingested. Ingestion of foods contaminated to this level can result in sporadic cases of illness as well as outbreaks. Whether or not outbreaks are detected depends on the number of persons who ingested the contaminated food and on the socio-cultural attitudes of the populace to report illness and the efficiency of a health agency to determine that the illness is foodborne and epidemiologically related to other cases. When numbers of pathogens insufficient to cause illness are ingested, an infected individual may become a carrier and may contaminate other foods that he touches.

Each step in the sequence of events necessary to contribute to outbreaks of foodborne disease is briefly reviewed.

(*a*) Microorganisms that cause foodborne disease are commonly associated with healthy persons or animals and the environment (e.g., soil), as well as with ill persons or animals. Chemicals that cause illness are frequently those used for acceptable agricultural or industrial practices but are applied too close to harvesting, used in a haphazard manner, or added to foods in excessive amounts.

(*b*) There are many sources of contamination of foods. Fruits and vegetables can become contaminated during production on farms, and seafoods and water-grown vegetables can become contaminated in their aquatic

environment. Meat can become contaminated if the meat is taken from an animals that has septicemia, if infectious lesions remain on sites used as meats, if edible organs are infected or inedible infected organs leak on meat surfaces, or if cross-contamination from animal feces occurs. Eggs become contaminated during formation, while in contact with nesting material, or by droppings. Foods are also contaminated by workers and by environmental sources during handling and preparation in food processing plants, food service establishments, and homes. The point at which contamination occurs depends on the natural sources of a pathogen or the source of a poisonous substance and on the opportunities for transfer at each stage of the food production-processing-preparation chain.

(*c*) Pathogenic bacteria on and in the contaminated food will multiply if the food contains sufficient moisture and sufficient quantity and variety of nutrients; if the pH and the redox potential of the food are favorable; if food-holding temperatures are within the growth range (particularly when the temperature is near the optimal growth temperature for the particular kind of pathogenic bacteria for enough time), and if they can successfully compete with the mixed microbial flora on and in the foods. Growth of spoilage bacteria that are usually on raw foods often inhibits growth of pathogenic bacteria. Raw foods, however, become vehicles of foodborne outbreaks when the ratio of pathogen to competitive flora is high or when the contaminating strain of pathogen is particularly virulent or is able to survive the competition of other flora, the effects of processes, and multiply during storage.

For heat-processed foods to become vehicles of foodborne disease outbreaks, pathogens — particularly bacterial spores — survive heat processing or the food becomes contaminated after the heat processing. Pathogenic bacteria that survive processing and preparation usually have to multiply to reach levels that cause disease. Most vegetative bacteria — but not spores — will be killed if during cooking any contaminated portions of food reach 73.9 C (165 F) for a few seconds (or even temperatures as low as 60 C (140 F), if held at such temperatures long enough. All foods, however, are not cooked before being eaten, and the temperatures reached during cooking can be too low or the time too short (at that temperature) to kill pathogens.

Pathogens often get into food preparation environments on contaminated raw foods, particularly raw foods of animal origin. These organisms are killed if food is thoroughly cooked; but, before the food is cooked, pathogens can contaminate hands of workers who touch the raw foods, and they can contaminate equipment that is used in their processing, preparation, or storage. These same foods after heat processing become recontaminated or other foods can become contaminated if they are handled by these workers or processed or prepared with the same equipment which has not been subsequently cleaned and sanitized.

A food contaminated with foodborne disease bacteria can support their growth under certain conditions: the food must contain sufficient moisture and essential nutrients and be within favorable pH and redox ranges to support growth of these pathogens; it must be kept within a temperature range that permits these bacteria to multiply (this is usually near the organism's optimal temperature for growth); and the food must be kept at such temperatures long enough for sufficient organisms (or toxins) to be produced to cause illness in those who ingest the food.

(*d*) Ingestion of contaminated food, however, does not always result in illness. Enough pathogens must be swallowed to exceed a person's threshold of resistance if illness is to result. Adult human volunteer feeding studies have indicated susceptibility resistance threshold levels for various enteric pathogens (*10*). Ten *Shigella dysenteriae* 1, 180 *Shigella flexneri* 2a, or 1,000 *Vibrio cholerae* biotype inaba can, if ingested, cause illness or carrier status. These quantities could conceivably be present on foods that were contaminated in fields or in watercourses by irrigation water containing sewage, or that were contaminated during handling by infected persons. Ten thousand *Salmonella typhi* and *V. cholerae* biotype inaba can cause illness; conceivably this amount could be present on foods recently fertilized with night soil or raw sewage. Most of the other organisms (such as *Salmonella*, *Clostridium perfringens*, and *Escherichia coli*) for which human feeding tests have been carried out usually require time for multiplication before the large number (often 100,000 or more) necessary to cause illness would be generated (*10*). Staphylococcal enterotoxin is produced when enterotoxic strains of *Staphylococcus aureus* multiply; there are usually 500,000 or more staphylococci present per gram before enterotoxin is detectable (*9*). Infants, elderly persons, malnourished persons, and persons with concomitant illness are more susceptible than healthy adults; perhaps a log or more reduction in dosage would cause illness among them.

It is unlikely that an infective dose of salmonellae would be on lettuce or other raw vegetables, for example, but it is possible for the same foods to contain an infective dose of shigellae. Shigellae, however, do not usually survive long in the microbiologically competitive environment of food. Salmonellae, *C. perfringens*, and many other foodborne disease causing bacteria would be problems if contaminated foods were allowed to stand at room temperature, be stored in hot-holding devices at bacterial incubating temperatures, or be stored in large pots or otherwise in bulk in refrigerators for sufficient time to permit them to multiply to sufficient numbers to cause illness.

Each sequence of events described is shown to be an important factor that contributes to foodborne disease outbreaks by critical review of epidemiologic investigations and surveillance reports for the period 1961 through 1976.

MATERIALS AND METHODS

The present study, as well as those of the past (5-7), was done by gathering details on operational or constructional factors that contributed to outbreaks of foodborne illness which were reported in public health literature or national surveillance data. Information was sought from periodic surveillance reports that are published by the Center for Disease Control (CDC); articles in journals; reports of outbreaks that have been sent to CDC from states, local health departments, or Federal agencies; and reports from CDC's Epidemic Intelligence Service. The surveillance reports that were previewed are *Morbidity and Morality Weekly Reports* (24) *Salmonella Surveillance Reports* (25), *Shigella Surveillance Reports* (27), *Hepatitis Surveillance Reports* (26), and *Trichinosis Surveillance Reports* (28). *Index Medicus* (1961-1977), *Excerpta Medica* (1961-1977), *Abstracts of Hygiene* (Bulletin of Hygiene), (1961-1977) and *Current Bibliography of Epidemiology* (1961-1977) were reviewed for listings of articles concerning foodborne disease outbreaks that occurred in the United States. Only those outbreaks that contained some mention of a food production, processing or preparation history were included in the data. Summary data were only used if there was a narrative to confirm the contributing factor. Outbreaks cited by one source were checked against the other source to avoid duplication. This survey, although pointing out many important factors that contribute to outbreaks, suffers certain shortcomings, which include: inadequate reporting of outbreaks; and incomplete reporting, write-up, or abstracting of these factors and outbreaks.

RESULTS AND DISCUSSION

In the United States, factors that have been frequently shown to contribute to outbreaks of foodborne disease are (in order of frequency of occurrence): inadequate cooling of foods, lapse of a day or more between preparing and serving, infected persons having touched foods which are not subsequently heat-processed, inadequate time or temperature or both during heat processing of foods, insufficiently high temperature during hot storage of foods, inadequate time or temperature or both during reheating of previously cooked foods, ingesting contaminated raw foods or raw ingredients, and other factors listed in Table 1. This table compares the most frequently reported factors that contributed to the occurrence of foodborne disease outbreaks for three periods, 1961 through 1970 (5) 1971 and 1972 (6, 7), and 1973 through 1976. A more detailed breakdown of the factors that have been shown to contribute to specific outbreaks of foodborne disease reported from 1973 through 1976 is shown in Table 2.

Inadequate cooling practices are the major contributors to outbreaks of foodborne disease. Data from these outbreaks that were reported in the U.S. from 1973 through 1976 show that the following practices led to inadequate cooling: leaving foods at room temperature for several hours, storing foods in large pots or pans or otherwise in bulk in refrigerators, storing foods in refrigerators that maintain temperatures above recommended levels or permit temperatures above recommended levels because of malfunction, nonrefrigerated transport in trucks or car trunks, and storing in ovens that were turned off (Table 3).

Data from the United States can be compared to a summary of some outbreaks of foodborne disease that have occurred in England and Wales and summarized in the British Medical Journal from 1969 to 1976 (Table 4).

TABLE 1. *The most important factors that contributed to the occurrence of 1,152 outbreaks of foodborne disease (by rank and percent)*[1] *reported in public health literature or surveillance data during 1961 through 1976 and containing information about contributory factors.*

Contributory factor	1961-1970[2] (493)[4]	1971-1972[3] (232)	1973-1976 (427)	1961-1976 (1,152)
Inadequate cooling	1 (46)	1 (48)	1 (46)	1 (46)
Lapse of a day or more between preparing and serving	4 (21)	2 (23)	2 (20)	2 (21)
Infected person	3 (22)	4 (19)	3 (18)	3 (20)
Inadequate thermal processing, canning, or cooking	2 (24)	5 (10)	6 (11)	4 (16)
Inadequate hot storage	6 (13)	3 (21)	4 (16)	5 (16)
Inadequate reheating	7 (9)	6 (9)	5 (16)	6 (12)
Ingesting contaminated raw food or ingredient	5 (14)	7 (6)	7 (11)	7 (11)
Cross-contamination	7 (9)	7 (6)	10 (4)	8 (7)
Inadequate cleaning of equipment	9 (8)	9 (6)	8 (6)	9 (7)
Obtaining foods from unsafe sources	10 (6)	11 (3)	12 (4)[5,6]	10 (5)
Using leftovers	11 (3)	10 (4)	9 (5)	11 (4)

[1]More than 100% shows in percentage figures because multiple factors are usually necessary for outbreaks of foodborne disease to occur.
[2]Reference: Bryan (5)
[3]Reference: Bryan (7)
[4]Number of outbreaks reviewed in each survey.
[5]Storing high-acid foods in toxic containers ranked eleventh.
[6]Toxic species mistaken for edible varieties also ranked twelfth.

TABLE 2. *Inadequate cooling practices that contributed to 189 foodborne disease outbreaks reported 1973 through 1976.*

Inadequate practice	Number	Percent
Left at room temperature	105	56 (68)[1]
Stored in large container	43	23 (32)[1]
At room temperature in large container	18	10
Refrigerator unit above recommended temperatures	6	3 (6)[1]
At room temperature, then in refrigerator at above recommended temperatures	6	3
Transportation unit not refrigerated	5	3
Car trunk	3	2
Oven turned off	3	2

[1]Total percent when multiple poor practices occurred

In England and Wales, factors that have been shown to contribute to outbreaks of foodborne disease are (in order of frequency of occurrence): inadequate cooling of foods, lapse of a day or more between preparation and serving, inadequate time or temperature or both during reheating, inadequate time or temperature or both during cooking, infected persons having touched foods which are not subsequently cooked, ingestion of contaminated raw food or ingredient, obtaining foods from unsafe sources, insufficient high temperature during hot-holding, and other factors listed in Table 4. These factors are quite similar to the factors that contribute to foodborne outbreaks in the United States (Tables 1 and 2).

The factors that most often contribute to outbreaks of foodborne disease vary, depending on the type of establishment in which foods are handled. Annual foodborne disease surveillance data show that foods that are implicated in outbreaks are frequently mishandled in foodservice establishments and homes. For instance, from 1973-1976, 67.4% were mishandled in foodservice

TABLE 3. *Factors that contributed to the occurrence of 427 outbreaks of foodborne disease reported in public health literature and surveillance data during 1973 through 1976.*

Diseases	Factors affecting growth: Inadequate cooling	Inadequate hot storage	Inadequate thawing	Prepared a day or more before serving	Use of leftovers	Factors affecting survival: Growth during germination	Faulty fermentations	Factors affecting contamination: Inadequate cooking, thermal processing, or canning	Inadequate reheating	Infected person	Unsafe source	Contaminated raw ingredient	Toxic species mistaken for edible varieties	Cross-contamination	Inadequate cleaning of equipment	Selling contaminated products or toxic foods	Poor storage practices	Toxic containers	Incidental additives	Intentional additives	Unknown post-process contamination	Contamination by fertilizer or soil	Faulty sealing	Improper evisceration	Contaminated water	Mishandling	Number of outbreaks with data on factors that contributed to outbreaks
Bacterial																											
Staphylococcal intoxication	56 (2)[2]	10		30	1		1	3 (1)	(10)	29 (10)		(1)		1	4												69
Salmonellosis	25 (1)	8	1	10	3		2	6 (1)	14	3 (5)		14 (5)		11 (1)	11	1						1					61
Botulism	2	2					8	19 (3)	2														1				34
Clostridium perfringens gastroenteritis	18	9 (2)		13	4			3 (1)	15					(1)													24
Shigellosis	1			2	1				2	5 (2)																	7
Typhoid fever		1						1		1 (1)																	4
Brucellosis												3				1											4
Bacillus cereus gastroenteritis	2			1	1	1																					3
Vibrio parahaemolyticus gastroenteritis	2													2											1		3
Group A streptococcal infections	2			2						2																	2
Cholera	1						1				1	1															1
Scombroid poisoning	1																										1
Yersinia enterocolitica gastroenteritis																					1						1
Total Bacterial	110 (3)	30 (2)	1	58	10	1	12	32 (6)	33 (10)	40 (18)	1	18 (6)		14 (2)	15	2					1	1	1		1		214
Viral										10 (3)	2																
Hepatitis A																											15
Parasitic																											
Trichinosis								6				14 (1)		1	1												19
Anisakiasis												2															2
Toxoplasmosis												2															2
Amebiasis					1					(1)																	1
Diphyllobothriasis								1																			1
Total Parasitic					1			7		(1)		18 (1)		1	1												25
Toxic Plants																											
Mushroom poisoning											15		15														15
Pokeweek poisoning													2			1											2
Burdock root poisoning													1														1
Atropine poisoning																			1								1
Cyanide poisoning													(1)														1
Total Toxic Plants											15		18 (1)			1			1								20
Toxic Fish																											
Tetradon													1											1			1
Chemical																											
Copper poisoning					1													15									15
Monosodium glutamate poisoning																				6							6
Detergent (soap) phosphate poisoning																			3								3
Sodium nitrite																			2								2
Niacin																				2						2	2
Cadmium poisoning																		1									1
Fluoride poisoning																				1							1
Stropine poisoning																		1									1
Histamine poisoning							(1)																				1
Methyl propyl paraben poisoning																			1								1
Soy hypersensitivity																				1							1
Sulfuric acid poisoning																	1										1
Zinc poisoning																		1									1
Total Chemicals				1			(1)										1	18	6	10						2	36
Total diseases of known etiology	110 (3)	30 (1)	1	59	11	1	12 (1)	39 (6)	33 (10)	50 (22)	18	36 (7)	19 (1)	15 (2)	16	3	1	18	7	10	1	1		1	1	2	311
Percent[1]	35	10	<1	19	4	<1	4	14	14	23	6	14	6	5	5	1	<1	6	2	3				<1	<1	1	
Diseases of unknown etiology but vehicle identified	86 (1)	39	1	27	9			3 (1)	24	(6)		1 (1)		(1)	8												116
Total	196 (4)	69 (1)	2	86	20	1	12 (1)	42 (7)	57 (10)	50 (28)	18	37 (8)	19 (1)	15 (3)	24	3	1	18	7	10	1	1	1	1	1	2	427
Percent[1]	46	16	<1	20	5	<1	3	11	16	18	4	11	5	4	6	1	<1	4	2	2	<1	<1	<1	<1	<1	<1	

[1]More than 100% shows in percentage figures because multiple factors are usually necessary for foodborne disease outbreaks to occur.
[2]Data in parentheses represent factors that were suggested but not confirmed.

TABLE 4. *Factors that contributed to the occurrence of 81 outbreaks of foodborne disease, abstracted in the British Medical Journal, 1969 through 1976.*

Disease	Incubation				Process failure			Contamination							Number of outbreaks with data on contributory factors
	Inadequate cooling	Inadequate hot storage	Prepared a day or more before serving	Improper thawing	Inadequate cooking	Inadequate reheating	Inadequate thawing	Infected person touching cooked food	Unsafe source	Contaminated raw ingredient	Cross-contamination	Inadequate cleaning of equipment	Infected animals	Can seam opening	
Clostridium perfringens gastroenteritis	28 (93)[1]	3 (10)	24 (80)		5 (17)	15 (50)	2 (7)								30
Salmonellosis	16 (63)	4 (15)	14 (52)		11 (41)	6 (22)	1 (4)	1 (4)	8 (30)	11 (41)	7 (26)	5 (19)	1 (4)		27
Staphylococcal intoxication	15 (79)		12 (63)	1 (5)				11 (58)				1 (5)		4 (21)	19
Bacillus cereus gastroenteritis	2 (100)		1 (50)				1 (50)								2
Escherichia coli gastroenteritis		1 (50)			1 (50)	1 (50)			1 (50)	1 (50)			1 (50)		2
Group A streptococcal gastroenteritis	1 (100)		1 (100)					1 (100)							1
Total	62	8	52	1	17	23	3	13	9	12	7	6	2	4	81
Percent	(77)	(10)	(64)	(1)	(21)	(28)	(4)	(16)	(11)	(15)	(9)	(7)	(2)	(5)	

[1]More than 100% shows in percentage figures because multiple factors are usually necessary for outbreaks of foodborne disease to occur.

establishments, and 26.6% were mishandled in homes. (*29*). (Percentages were calculated with denominator of number of places where foods were known to be mishandled.)

The factors that occasioned outbreaks of foodborne disease when foods were mishandled in foodservice establishments in the United States during 1973 through 1976 are (in order of frequency of occurrence): inadequate cooling of foods, insufficiently high temperatures during hot-storage of foods, lapse of a day or more between preparing and serving food, infected person having touched foods which are not subsequently heat-processed, inadequate time or temperature or both during reheating of previously cooked foods, and other factors listed in Table 5.

Factors that occasioned outbreaks of foodborne disease when foods were mishandled in homes in the United States during 1973 through 1976 are (in order of frequency of occurrence): inadequate cooling, inadequate time or temperature or both during canning or cooking, mistaking toxic species of mushrooms and other plants for edible varieties, obtaining foods from unsafe sources, lapse of a day or more between preparing and serving, and other factors listed in Table 6.

Factors that have contributed to the occurrence of outbreaks of foodborne disease which resulted from foods mishandled in food processing plants are presented in Table 7 and have been reviewed previously (*6*).

Factors that contribute to outbreaks of foodborne disease form general patterns depending on the classification of the etiologic agent. Table 8 shows that factors that affect growth, survival, and contamination (all) frequently occur in outbreaks of bacterial foodborne illness. Factors that affect survival and contamination are of concern in outbreaks caused by parasites. Factors that affect contamination are of major concern in outbreaks caused by viruses, toxic plants, and chemicals.

Factors that contribute to outbreaks of any specific foodborne disease are unique for that disease. As data are collected over the years, patterns develop as indicated in Table 3-7 and Table 9. These can be studied and methods of prevention and control devised and priorities set.

Staphyloenterotoxicosis

Factors that most often contributed to outbreaks of staphylococcal food poisoning are inadequate cooling of foods, infected persons having touched cooked foods, and lapse of a day or more between preparing and serving. These factors and other less important factors are listed in Table 3-7 and Table 9.

Although *S. aureus* is sometimes isolated from raw meat and poultry, food workers or homemakers appear to be the main source of the organisms that contaminate foods which become vehicles of outbreaks of staphyloenterotoxicosis. Many persons carry *S. aureus* in their anterior nares; their hands readily become contaminated. Staphylococci are resident as well as transient on skin (*9*). Most persons who contaminate foods are nasal carriers; only occasionally does the contaminating organism come from cuts, burns, or boils (*2, 9, 19*).

Vehicles are usually foods that are rich in protein. Several particular amino acids and thiamine and niacin

TABLE 5. *Factors that contribute to the occurrence of 235 outbreaks of foodborne disease where mishandling occurred in foodservice establishments, 1973 through 1976.*

Diseases	Factors affecting growth					Factors affecting survival		Factors affecting contamination									Number of outbreaks with data on factors that contribute to outbreaks
	Inadequate cooling	Inadequate hot storage	Inadequate thawing	Prepared a day or more before serving	Use of leftovers	Inadequate cooking	Inadequate reheating	Infected person	Unsafe source	Contaminated raw ingredient	Cross-contamination	Inadequate cleaning of equipment	Contaminated water	Toxic containers	Incidental additives	Intentional additives	
Bacterial																	
Staphylococcal intoxication	38 (1)[2]	7		23	1	2 (1)	(7)	22 (7)		(1)	1	3					45
Salmonellosis	19 (1)	7	1	10	3	1	14	3 (3)		1 (1)	8 (1)	9					28
Clostridium perfringens gastroenteritis	12	8 (1)		9	3	3 (1)	11				(1)						18
Shigellosis	1			2			2	5 (1)									6
Vibrio parahaemolyticus gastroenteritis	2										2		1				3
Bacillus cereus gastroenteritis	2						1										2
Group A *Streptococcus* infections	2							2									2
Typhoid fever		1						1									2
Total Bacterial	76 (2)	25 (2)	1	47	9	6 (2)	28 (7)	33 (11)		1 (2)	11 (2)	12	1				106
Viral																	
Hepatitis A								8 (3)		2							13
Parasitic																	
Amebiasis				1				(1)									1
Toxoplasmosis										1							1
Trichinosis										1	1	1					1
Total Parasitic				1				(1)		2	1	1					3
Chemical																	
Copper poisoning														8			8
Monosodium glutamate poisoning																4	4
Detergent/soap/phosphate poisoning															3		3
Fluoride poisoning																1	1
Zinc poisoning														1			1
Total Chemical														9	3	5	17
Total diseases of known etiology	76 (2)	25 (2)	1	47	10	6 (2)	28 (7)	41 (15)	2	3 (2)	12 (2)	13	1	9	3	5	139
Percent[1]	56	19	1	34	7	6	25	40	1	4	10	9	1	6	2	4	
Diseases of unknown etiology but vehicle identified	72 (1)	37	1	21	6	3 (1)	24	(5)			(1)	8					96
Total	148 (3)	62 (2)	2	68	16	9 (3)	52 (7)	41 (20)	2	3 (2)	12 (3)	21	1	9	3	5	235
Percent[1]	63	27	1	29	7	5	25	26	1	2	6	9	<1	4	1	2	

[1]More than 100% shows in percentage figures because multiple factors are usually necessary for foodborne disease outbreaks to occur.
[2]Data in parentheses represent factors that were suggested but not confirmed.

TABLE 6. *Factors that contributed to the occurrence of 122 outbreaks of foodborne disease where mishandling occurred in homes, 1973 through 1976.*

Diseases	Factors affecting growth: Inadequate cooling	Inadequate hot storage	Prepared a day or more before serving	Use of leftovers	Faulty fermentations	Factors affecting survival: Inadequate cooking or canning	Inadequate reheating	Factors affecting contamination: Infected person	Unsafe source	Contaminated raw ingredient	Toxic species mistaken for edible varieties	Cross-contamination	Inadequate cleaning of equipment	Poor storage practices	Faulty sealing	Toxic containers	Improper evisceration of toxic fish	Intentional additives	Number of outbreaks with data on factors that contributed to outbreaks
Bacterial																			
Botulism	1	2			7	18 (1)	2								1				30
Salmonellosis	6	1				2 (1)		(1)		7 (4)		3	1						20
Staphylococcal intoxication	12 (1)[2]	2	6				(2)	4 (3)											15
Clostridium perfringens gastroenteritis	6	1	4	1			3												7
Typhoid fever						1		(1)											2
Brucellosis										1									1
Cholera	1				1				1	1									1
Shigellosis								(1)											1
Total Bacterial	26 (1)	6	10	1	8	21 (2)	5 (2)	4 (6)	1	9 (4)		3	1		1				72
Viral																			
Hepatitis A																			2
Parasitic																			
Trichinosis						5				12 (1)									16
Anisakiasis										2									2
Diphyllobothriasis						1													1
Toxoplasmosis										1									1
Total Parasitic						6				15 (1)									20
Toxic Plants																			
Mushroom poisoning									15		15								15
Burdock root poisoning											1								1
Cyanide poisoning											(1)								1
Pokeweed poisoning											1								1
Total Toxic Plants									15		17 (1)								18
Toxic Fish																			
Tetradon											1						1		1
Chemical																			
Copper poisoning																4			4
Monosodium glutamate poisoning																		2	2
Cadmium poisoning																1			1
Lead poisoning																1			1
Sulfuric acid poisoning														1					1
Total chemical														1		6		2	9

Total disease of know etiology	26 (1)	6	10	1	8	27 (2)	5 (2)	4 (6)	16	24 (5)	18 (1)	3	1	1	1	6	1	2	122
Percent[1]	22	5	8	1	6	24	6	8	13	24	16	2	1	1	1	5	1	2	
Diseases of unknown etiology but vehicle identified	15	2	5	4				(1)		2									19
Total	41 (1)	8	15	5	8	27 (2)	5 (2)	4 (7)	16	26 (5)	18 (1)	3	1	1	1	6	1	2	141
Percent[1]	30	6	11	4	6	21	5	8	11	22	13	2	1	1	1	4	1	1	

[1]More than 100% shows in percentage figures because multiple factors are usually necessary for foodborne outbreaks to occur.
[2]Data in parentheses represent factors that were suggested but not confirmed.

TABLE 7. *Factors that contributed to foodborne outbreaks where mishandling occurred in food processing plants.*

	Factors affecting growth				Factors affecting contamination							
Diseases	Inadequate cooling	Growth during germination of seed and development of plant	Faulty fermentations	Inadequate heat processing	Infected person	Contaminated raw ingredient	Inadequate cleaning of equipment	Contamination by fertilizer and soil	Incidental additives	Intentional additives	Unknown post-process contamination	Number of outbreaks with data on factors that contributed to outbreaks
Bacillus cereus gastroenteritis		1										1
Botulism	1		1	1(2)								4
Brucellosis						2						2
Salmonellosis			2	3	(1)	6	1	1				12
Staphylococcal intoxication	4		1	1	2		1					7
Yersinia enterocolitica gastroenteritis											1	1
Trichinosis				1								1
Chemical			(1)						2	1		4
Total	5	1	4 (1)	6(2)	2(1)	8	2	1	2	1	1	32
Percent	16	3	16	25	9	25	6	3	6	3	3	

TABLE 8. *Percent[1] of factors that contributed to the occurrence of outbreaks of several classes of foodborne diseases reported in public health literature or surveillance data during 1961 through 1976.*

Factor	Diseases				
	Bacterial (707)[2]	Virai (44)	Parasitic (72)	Toxic plants (44)	Chemical poisonings (79)
Factors affecting growth					
Inadequate cooling	55				
Inadequate hot storage	16				
Inadequate thawing	<1				
Growth during germination	<1				
Faulty fermentation	2				1
Lapse of day or more between preparing and serving[3]	26				
Use of leftovers[3]	4		1		1
Factors affecting survival					
Inadequate cooking, thermal processing, or canning	20	2	59		
Inadequate reheating	14				1
Factors affecting contamination					
Infected persons	25	66	1		
Unsafe source	1	36		52	
Contaminated raw ingredient	13		64		
Toxic species mistaken for edible varieties				66	
Cross contamination	9		8		
Inadequate cleaning of equipment	8		8		
Toxic containers					47
Incidental additives				9	15
Intentional additives					34
Selling contaminated products or toxic foods	<1			2	
Poor storage practices					6
Contamination of washed dishes		2			
Unknown post-processing contamination	<1				
Contamination by fertilizer or soil	1				
Faulty sealing of cans or jars	<1				
Improper evisceration	<1				
Contaminated water	<1				
Misbranding					3

[1]Percentages tabulated for each class of diseases separately. More than 100% show in percentage figures because multiple factors are usually necessary for outbreaks of foodborne disease to occur.

[2] Number of outbreaks on which data are based.

[3]Indirect factors that influence situation only when other factors occur.

are necessary for the growth of staphylococci and production of enterotoxin. Many of the vehicles (such as (custard or ham) contain relatively high concentrations of sugar or salt; thus, they have a lower water activity than is optimum for most bacteria. Such substances inhibit the growth of many bacteria, but not staphylococci (*8, 9*). Foods that have been implicated in outbreaks of staphylococcal food poisoning have usually been cooked and then cut, sliced, mixed, grated, ground, or otherwise handled by persons who are carriers of enterotoxigenic strains of *S. aureus* (*9, 16*). The cooking is itself often an important factor that indirectly contributes to outbreaks because *S. aureus* does not compete successfully with the bacteria that are commonly found in raw foods. Cooking reduces or eliminates the nonsporeforming bacterial flora, including staphylococci. After cooked foods are subsequently handled and thus contaminated, they must remain at room temperature or be refrigerated in such bulk as to retain heat for a sufficient time for *S. aureus* to multiply and produce enterotoxin in the absence of numerous competitive organisms. Staphyloenterotoxins are not destroyed by the usual reheating in routine food processing and service operations. (*9*).

Salmonellosis

Factors that have most often contributed to outbreaks of salmonellosis are inadequate cooling of foods, ingesting contaminated raw foods or ingredients, inadequate time or temperature or both during heat processing of foods, cross-contamination from raw foods (frequently raw meat, raw poultry, eggshells, and unpasteurized egg products) to cooked foods, lapse of a day or more between preparing and serving, inadequate cleaning of equipment, insufficiently high temperatures during hot storage, infected person having touched foods which are not subsequently heat processed, and inadequate time or temperature or both during reheating of previously cooked foods. These factors and other less frequently reported factors are listed in Tables 3-7 and Table 9.

Current epidemiologic evidence indicates that meat-

TABLE 9. *Percent*[1] *of factors that contributed to the occurrence of outbreaks of several bacterial foodborne diseases reported in public health literature or surveillance data during 1961 through 1976.*

Factor	Bacterial Diseases						
	Salmonellosis (238)[2]	Staphylococcal intoxication (214)	*Clostridium perfringens* gastroenteritis (93)	Botulism (85)	Shigellosis (27)	Typhoid fever (14)	*Vibrio parahaemolyticus* gastroenteritis (12
Factors affecting growth							
Inadequate cooling	47	78	76	13	56	7	67
Inadequate hot-holding	14	18	46	2		7	
Lapse of day or more between preparing and serving	17	44	51		22		
Use of leftovers	4	3	12		4		
Faulty fermentations	1			9			
Factors affecting survival							
Inadequate cooking, heat processing, canning	21	3	(9)[3]	80		7	
Inadequate reheating	13	(7)[3]	45	2	7		
Factors affecting contamination							
Infected person	13	53			89	79	
Contaminated raw ingredients	32						42
Cross-contamination	21	3	2				33
Inadequate cleaning of equipment	15	9	1				
Unsafe source	1					14	17
Contaminated water							8

[1]Percentages tabulated for each group of diseases separately. More than 100% shows in percentage figures because multiple factors are usually necessary for outbreaks of foodborne disease to occur. Factors occurring in less than 1% of outbreaks omitted.
[2]Number of outbreaks in which data are based.
[3]Poor practices reported but probably would not have altered outcome of outbreak.

source animals are major reservoirs of salmonellae. Animals become infected either from feed which frequently contains salmonellae or from their environment which has been contaminated by previous flocks or herds. The infection rate increases when swine or cattle are transferred to slaughter and held in pens. A few infected (or superficially contaminated) animals are the source of salmonellae that are spread from fecal matter to many carcasses. Considerable contamination occurs early in processing, during defeathering of fowl or dehairing of swine. Washing reduces the level of contamination, but subsequent handling enhances the possibility of cross-contamination, which increases the number of contaminated carcasses. The primary sources of salmonellae in retail stores, food processing establishments, and homes are poultry carcasses, cuts of meat, and unwashed eggshells. Consequently, poultry, meat, and eggs have been more commonly associated with outbreaks than have other foods (*2, 12, 23*).

Foods that have been vehicles in outbreaks of salmonellosis usually become contaminated by salmonellae in one of the following ways: (*a*) animal and fowl carcasses are contaminated with fecal matter during processing; (*b*) foods that receive no further heat treatment or that are inadequately heated are contaminated by raw ingredients, such as checked or cracked raw eggs that already contain salmonellae or have them on their shells; (*c*) cooked foods become contaminated by touching unwashed or inadequately cleaned equipment that has been used to process contaminated raw foods of animal origin or by persons who have previously handled such contaminated foods (cross-contamination); and, to a lesser extent (*d*) human carriers who practice poor personal hygiene. It has been difficult to determine during outbreak investigations whether infected food workers were carriers and the source of contamination or whether they had eaten the same foods as did the other persons who had become ill or had handled the contaminated food.

To become dangerous, foods contaminated by salmonellae usually must be held long enough at suitable temperatures for salmonellae to multiply to sufficient numbers to cause infection. In most episodes of salmonellosis, the ill have ingested millions of salmonellae. But smaller numbers of some serotypes can apparently cause illness in susceptible persons (*1, 10, 13, 17*).

Clostridium perfringens gastroenteritis

Factors that most often contributed to outbreaks of *C. perfringens* gastroenteritis are inadequate cooling of foods (meat, poultry, gravy, stock), lapse of a day or more between preparing and serving, inadequate time or temperature or both during hot storage of foods, and inadequate time or temperature or both during reheating of previously cooked foods. These and other less important factors are listed in Tables 3-7 and Table 9.

C. perfringens is commonly found in the intestinal tract of man and animals and in dust and soil (*15*). During processing, meat and poultry frequently become contaminated by vegetative cells or spores of *C. perfringens* from one or more of these sources. Spores of some strains of *C. perfringens* survive boiling for 4 to 6 h; so, they survive the time-temperature effects of most cooking operations. Furthermore, cooking drives off oxygen; this can reduce the redox potential of the food to a level at which *C. perfringens* can initiate growth. Cooking also kills competitive organisms and heat-shocks any *C. perfringens* spores (that are present so that a greater number of *C. perfringens* spores) germinate than would occur without heating. Because this organism is commonly found in the environment of or on equipment in foodservice establishments, as well as in the intestinal tracts and on the hands of workers, foods can easily become contaminated after cooking (*11*). Spores that survive cooking must germinate and vegetative cells that evolve or that have contaminated cooked foods must multiply to sufficient numbers in the food to cause illness when ingested.

C. perfringens can grow only if several particular amino acids and vitamins are available to it. Few foods, other than meat and poultry (or their stock or gravy) contain the necessary nutrients. *C. perfringens* will multiply in a protein-rich substrate when such foods are stored for several hours at room temperature, stored in large pots in a refrigerator, or stored in a warming device that holds the food at 50 C or below (*4, 14, 15*).

Botulism

Outbreaks of botulism result when *Clostridium botulinum* survives the effects of time-temperature exposure during thermal processing, and when this organism multiplies and produces neurotoxin in the anaerobic environment of cans, jars, plastic bags, or bulk food (Table 3, 6, and 8). Inadequate time or temperature or both during cooking (as the failure to process hot-packed jars in pressure cookers) of home-preserved foods still contributes to most outbreaks. Other outbreaks, however, have been traced to fermented, smoked, and dried foods which are inadequately processed. Sources of *C. botulinum* are soil, mud, or water from which foods are grown and harvested (*21, 22*).

Vibrio parahaemolyticus gastroenteritis

Vibrio parahaemolyticus is found in high numbers in warm sea waters, and is frequently isolated from raw marine seafoods (*18, 20*). Several factors usually contribute to outbreaks (Tables 3, 5 and 9). Inadequate refrigeration has contributed to multiplication of *V. parahaemolyticus*. Inadequate time or temperature or both during cooking has allowed these organisms to survive in seafoods. Handling raw seafoods and then cooked foods and processing them on the same equipment have recontaminated the cooked foods.

Other foodborne diseases

In outbreaks of shigellosis, typhoid fever, and streptococcal pharyngitis, foods are usually contaminated by infected workers who practice poor personal hygiene (Table 3-7, and Table 9). The contaminated

food frequently is either not refrigerated or inadequately refrigerated. Occasionally the practice of obtaining foods from unsafe sources (e.g., shellfish from contaminated bays) has led to outbreaks of typhoid fever (*3*). Foodborne hepatitis A occurs either after sewage contaminates waters from which oysters or clams (which are eaten raw) are harvested or after infected persons touch foods which are not subsequently heat-processed. Factors that contribute to outbreaks of trichinosis are either inadequate time or temperature or both during cooking or ingestion of raw pork (and sometimes other meat such as bear meat and rarely beef or mutton if cross-contamination from infested pork occurred in grinding machines).

CONCLUSIONS

Factors that contribute to the occurrence of foodborne disease which are derived from epidemiologic data are compatible with information that is derived from the study of the ecology, biology, and toxicology of etiologic agents of foodborne disease. This information should be used to plan and direct food protection programs and to set priorities based on prevention of contemporary disease problems.

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Factors contributing to outbreaks of food poisoning in England and Wales 1970–1979

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SUMMARY

Epidemiological data accompanying reports of more than 1000 outbreaks of food poisoning have been studied to determine the factors which most frequently contributed to the outbreaks. Preparation of food in advance of needs combined with improper storage and inadequate cooking, cooling and reheating were the most common factors. Infected food handlers did not play a significant role except in instances of *S. aureus* food poisoning.

INTRODUCTION

Published reports on the incidence of bacterial food poisoning and salmonella infection in England and Wales give information on the number of outbreaks and cases, location, causal agent and type of food as well as details of outbreaks of special interest and prevalence of particular serotypes and phage types of the organisms implicated. There is available less information on the malpractices in food preparation processes that lead to food poisoning. In 1978 Bryan analysed the factors contributing to 81 outbreaks reported in the United Kingdom from 1969 to 1976. The information was abstracted from reports in the British Medical Journal and only represented a small proportion of the outbreaks during that period. Other surveys on factors contributing to outbreaks have been carried out in the United States (Bryan, 1972, 1975, 1978). The present analysis includes data from more than 1000 outbreaks reported between 1970 and 1979 and includes many of the outbreaks analysed by Bryan.

MATERIALS AND METHODS

The data have been extracted from routine reports of general and family outbreaks of food poisoning made to the PHLS Communicable Disease Surveillance Centre (CDSC) by laboratories and local authorities in England and Wales. These have been supplemented by published reports and from correspondence accompanying cultures of *Clostridium perfringens*, *Staphylococcus aureus* and *Bacillus cereus* sent to the Food Hygiene Laboratory for serotyping and for toxin testing; and also from questionnaires on food preparation and storage procedures

Table 1. *Distribution of general and family outbreaks of food poisoning analysed by aetiological agent*

Causal agent	Number of outbreaks
Salmonella spp.	396
C. perfringens	387
S. aureus	133
B. cereus and other *Bacillus* spp.	53
Other-bacterial	
V. parahaemolyticus	8
E. coli	3
C. botulinum	1
Y. enterocolitica	1
Other-non-bacterial	
scombrotoxin	47
red kidney beans	7
virus	1
Not known	7
Total	1044
Total outbreaks reported 1970–9 (Anon 1980)	6457

which the laboratory sends out when insufficient information is received with cultures of *C. perfringens* from outbreaks.

The outbreaks studied were mostly due to bacteria, i.e. *Salmonella* spp., *C. perfringens*, *S. aureus*, *B. cereus*, other *Bacillus* spp., *Vibrio parahaemolyticus*, *Escherichia coli*, *C. botulinum* and *Yersinia enterocolitica*. Other episodes included scombrotoxic fish poisoning, red kidney bean poisoning and viral gastroenteritis. The reports analysed varied in the amount of detail furnished concerning the history of ingested food, with one factor recorded in some instances and as many as 5 or 6 in others. It is likely that when only single factors were recorded other factors were involved but, clearly, these could not be included in the analysis.

RESULTS

Between 1970 and 1979 sufficient data were obtained from 1044 general and family outbreaks of food poisoning to include in the analysis (Table 1). Over this period there were 6457 such outbreaks reported to the PHLS (Anon, 1980) and although some of the outbreaks studied were not included in this figure it is still apparent that in only a small proportion of outbreaks was adequate epidemiological data supplied. In 67 % of the outbreaks studied (609 of 1044) the food was prepared in restaurants, hotels, clubs, hospitals, institutions, schools and canteens and in only 20 % (206 of 1044) was food prepared in family homes (Table 2). In 817 (78 %) of the outbreaks the food consumed was meat (444, 42 %) or poultry (373, 36 %). Of the remaining 227 outbreaks the food incriminated varied with the agent involved. In outbreaks of salmonellosis the principal food vehicle apart from meat

Table 2. *Place of consumption or origin of food incriminated in* 1044 *general and family outbreaks of food poisoning in England and Wales* 1970–1979

Place or function	*Salmonella* spp.	*C. perfringens*	*S. aureus*	*B. cereus* and other *Bacillus* spp.	Other-bacterial	Other-nonbacterial	Not known	Total (%)
Restaurants, hotels, clubs, holiday camps	83	48	13	27	4	1	3	179 (17·1)
Hospitals	28	80	6	1	—	—	—	115 (11·0)
Banquets, dinners, receptions, parties	90	18	16	—	2	—	1	127 (12·2)
Institutions	9	73	14	—	1	—	—	97 (9·3)
Schools	13	62	15	1	1	—	—	92 (8·8)
Canteens, meals-on-wheels	7	76	5	—	—	—	1	89 (8·5)
Shops, bakeries, take aways	36	1	22	20	—	1	—	80 (7·7)
Farms	43	—	—	—	1	—	—	44 (4·2)
Outings, holiday parties	3	4	2	—	—	1	—	10 (1·0)
Ships, aeroplanes	1	—	—	—	3	—	—	4 (0·4)
Not known	—	—	1	—	—	—	—	1 (0·1)
Family homes	83	25	39	4	1	52	2	206 (19·7)
Total	396	387	133	53	13*	55†	7	1044 (100)

* *V. parahaemolyticus* (8), *E. coli* (3), *C. botulinum* (1), *Y. enterocolitica* (1).
† Scombrotoxin (47), red kidney beans (7), virus (1).

and poultry was raw milk while most of the *B. cereus* outbreaks were associated with rice.

Of the factors which most commonly contributed to outbreaks in England and Wales (Table 3), preparation of the food more than half a day in advance of needs occurred in more than 60%. Other major contributing factors were storage at ambient temperature (40%), inadequate cooling (32%), inadequate reheating (29%) and the use of contaminated processed food (19%). The latter included such foods as cooked meats and poultry, pies and take-away meals prepared in premises other than those in which the final dish was consumed but did not include canned foods.

Infected food handlers did not play a significant role except in instances of *S. aureus* food poisoning. In many of these (41 of 44) the same phage and enterotoxin producing type of *S. aureus* was isolated from both food handler and food. Although infected food handlers were recorded in 126 salmonella outbreaks, in only 9 (7%) was there evidence to suggest that they were the original source of the contaminating organism. In two of the outbreaks the food handlers had recently

Table 3. *Factors contributing to* 1044 *outbreaks of food poisoning in England and Wales* 1970–1979 *by aetiological agent*

	Number of outbreaks in which factor recorded							
Contributing factor	*Salmonella* spp.	*C. perfringens*	*S. aureus*	*B. cereus* and other *Bacillus* spp.	Other-bacterial	Other-nonbacterial	Not known	Total (%)
Preparation too far in advance	173	338	66	49	4	—	3	633 (60·6)
Storage at ambient temperature	115	208	54	32	1	2	1	413 (39·6)
Inadequate cooling	71	236	10	16	—	—	—	333 (31·9)
Inadequate reheating	47	215	4	29	3	—	2	300 (28·7)
Contaminated processed food (excluded canned)	105	9	25	4	9	46	1	199 (19·1)
Undercooking	91	62	1	1	1	4	1	161 (15·4)
Inadequate thawing	42	22	—	—	—	—	—	64 (6·1)
Cross contamination	57	3	2	—	—	—	—	62 (5·9)
Improper warm holding	9	42	—	8	1	—	—	60 (5·7)
Infected food handlers	9	—	44	—	1	—	—	54 (5·2)
Use of left overs	21	20	8	1	—	—	—	50 (4·8)
Raw food consumed	37	—	—	—	1	8	—	46 (4·4)
Extra large quantities prepared	13	17	2	—	—	—	—	32 (3·1)
Contaminated canned food								
(*a*) freshly opened	2	3	16	1	1	3	3	29 (2·8)
(*b*) not freshly opened	—	—	8	—	1	—	—	9 (0·9)
(*c*) not known	—	—	7	—	—	—	—	7 (0·7)
Total	792	1175	247	141	23	63	11	2452 factors

returned from holidays in Spain, while in the other seven the food handlers had continued to prepare food while suffering symptoms of gastroenteritis. In most instances food handlers are victims, not sources, and become infected either from frequent contact with contaminated raw food, from tasting during preparation or from eating left over contaminated cooked food.

Although in most types of food poisoning preparation in advance and storage at ambient temperature were the two main factors involved (Table 3) certain factors appeared to be associated with particular organisms. In outbreaks due to salmonellas use of contaminated processed food (27%), undercooking (23%) and inadequate cooling (18%) were the next most commonly involved factors. Con-

taminated processed food was associated with more outbreaks caused by *Salmonella* spp. than any of the other types of bacterial food poisoning. In outbreaks due to *C. perfringens* multiple factors were frequently recorded with inadequate cooling (61 %) and inadequate reheating (56 %) figuring prominently together with preparation in advance (87 %) and storage at ambient temperature (54 %). Infected food handlers (33 %) and contaminated processed food (19 %) were important factors in outbreaks due to *S. aureus*; a significant proportion of these outbreaks were attributed to the consumption of canned food (23 %) of which more than half were reported as being freshly opened just prior to consumption.

Of the 53 outbreaks due to *Bacillus* spp. 45 of the 52 caused by *B. cereus* followed the consumption of rice at restaurants or from take away shops. One outbreak attributed to *B. licheniformis* was associated with a commercially produced meat pie. The factors contributing to outbreaks were similar to those associated with *C. perfringens*.

Other food poisoning outbreaks

Of the 13 outbreaks studied (Table 1) due to bacteria other than those mentioned above, nine were associated with fish and shellfish (*V. parahaemolyticus* and *C. botulinum*), one with coleslaw (*Y. enterocolitica*), two with meat products (*E. coli*) and one with raw milk (*E. coli*). In this small group of outbreaks processed food contaminated with the agent or its toxin was the most frequently implicated factor. The one episode of botulism was caused by canned salmon from Alaska which contained *C. botulinum* type E (Ball *et al.* 1979). The can was shown to have a small defect through which the *C. botulinum* entered after the product was processed.

There were 55 outbreaks (Table 1) in which the cause of illness was a non-bacterial toxin. In those due to red beans, the toxin (red bean haemagglutinin) occurs naturally in the food itself whereas in those due to scombrotoxin, the toxin is produced by bacteria acting on the food. In one large outbreak the infectious agent was a virus (Appleton & Pereira, 1977).

In seven outbreaks various contributing factors were reported but a causal agent was never identified. Three of these were associated with freshly opened canned foods.

DISCUSSION

In the sequence of events which occurs when persons succumb to bacterial food poisoning, one or more factors may cause the initial contamination in a food item to become an infectious or intoxicating dose. The initial dose of organisms in a food is usually insufficient to cause food poisoning but the presence of sufficient nutrients, moisture and warmth, together with a time lapse before consumption may allow the microorganisms to reach harmful levels. In the case of *C. perfringens*, multiplication can be extremely rapid as this organism has a high optimum temperature for growth (43–47 °C) and a short generation time of about 12 min. Other characteristics of the food that may also play a part in allowing bacterial multiplication are pH, water activity (a_w), redox potential and levels of other organisms which may compete with or inhibit the growth of potential pathogens or both.

This analysis shows that although there are a number of factors which continually contribute to outbreaks there is a wide variety of other factors which play a part, depending on the organism involved and the type of food consumed. For example the food most frequently implicated in the outbreaks of salmonella food poisoning studied was poultry. A recent survey showed that 79 of 100 frozen chickens purchased from normal retail outlets contained salmonellas, some containing more than one serotype (Communicable Disease Surveillance Centre, 1980). The procedures carried out from thawing a frozen bird to serving the cooked meat offer many opportunities for the spread of contamination via hands, equipment and surfaces and for the survival and multiplication of salmonellas if there is not strict control of hygiene and temperature of food storage at all stages. Thus in reports of outbreaks factors such as inadequate thawing, undercooking and cross contamination are important contributory factors.

In 168 outbreaks (123 *Salmonella* spp., 45 *C. perfringens*) the type of poultry implicated was turkey, often large ones (25 lbs or more) and frozen. These birds are a particular problem because their size creates difficulties in thawing, cooking, cooling and storing adequately to prevent growth of surviving or recontaminating organisms (Anon, 1974*a*, *b*; Noah, 1975). The increasing number of turkey associated outbreaks led to the issue of a Health Notice and Local Authority Social Services Letter on the Safe Preparation of Turkeys in 1977 (Department of Health and Social Security, 1977).

During cooking oxygen will be driven out of food so that an anaerobic environment is created. The heat-resistant spores of *C. perfringens* that survive most conventional cooking procedures will be heat activated and during any long slow cooling process there will be germination and rapid multiplication. As *C. perfringens* is widespread in the environment and is usually present in the animal intestine, it will be present naturally in most foods, particularly those of animal origin. Therefore prevention of this type of food poisoning depends almost entirely on temperature control during the whole process from raw to cooked food. This is well illustrated in Table 3, the four factors which were most frequently implicated in outbreaks of *C. perfringens* food poisoning all related to time and temperature at which the food was held prior to consumption.

The main source of *S. aureus* in relation to food poisoning outbreaks is the person preparing the food. The organism is frequently carried in the anterior nares, less commonly on the skin and, perhaps most importantly in relation to food poisoning, in septic lesions on the hands. Strains from any of these sites may be enterotoxigenic. The foods which act as vehicles of intoxication are usually those which have received much handling during preparation and which are consumed without further heat treatment, although the toxins are not necessarily inactivated even if the food is reheated. *S. aureus* can tolerate relatively high concentrations of salt and sugar so cured meats such as ham can provide an ideal environment for their growth while inhibiting other organisms and removing any competition.

B. cereus is a widespread naturally-occurring organism commonly found in cereals. Its spores can survive cooking so that when boiled rice is kept at kitchen temperature for frying on demand there is opportunity for germination and multiplication (Public Health Laboratory Service Working Party, 1976). The organisms produce enterotoxins and these can cause either an emetic or a

diarrhoeal syndrome; the outbreaks associated with rice have been the emetic type. The emetic toxin is very heat stable (Melling & Capel, 1978) and is not inactivated by frying. Prevention of this type of food poisoning depends on temperature control of the food and as *B. cereus* is similar to *C. perfringens* in that it produces heat resistant spores the factors contributing most frequently (Table 3) to both types of outbreaks are similar.

Although bacteria are involved in episodes of scombrotoxic fish poisoning they are not directly the infectious agent. Scombroid fish (tuna, mackerel, bonito) are rich in histidine and during storage at warm temperatures this is decarboxylated to histamine by the normal fish flora. Histamine is the toxic factor or is closely associated with the toxic factor and symptoms (principally a sharp or peppery taste in the mouth, hot flushing of the face and neck, sweating, bright red rash and headache with sometimes diarrhoea and nausea) are produced very rapidly after eating the fish (Gilbert *et al.* 1980). The toxin is very stable and cannot be destroyed by curing or heating so outbreaks can occur from canned fish (Murray, Hobbs & Gilbert, 1982). Scombrotoxic fish poisoning is probably beyond the control of the consumer, but is nevertheless preventable at the processing stage by ensuring that only fresh or frozen fish is used.

The outbreaks associated with red kidney beans were all due to consumption of the food in an uncooked or undercooked state. The toxin is inactivated by cooking the beans until soft (Noah *et al.* 1980).

This study has summarized data from outbreaks reported between 1970 and 1979. In 1980 a system was introduced by which Medical Officers for Environmental Health and Environmental Health Departments could report outbreaks to CDSC as part of the rationalisation of food poisoning surveillance. The new report forms have been useful in documenting factors contributing to outbreaks in more detail and more consistently than is possible in laboratory reports (Anon, 1981). However, even with the new report forms information on contributory factors was provided in only some of the outbreaks (31 % *Salmonella* spp., 74 % *C. perfringens*).

Food poisoning statistics for Scotland in 1980 (Collier *et al.* 1981) also give some information on factors involved in outbreaks, although, as with England and Wales, data were only available for a proportion of the outbreaks reported (44 of 147 general and family outbreaks). The factors recorded were similar to those described in the present study although the main factors, preparation in advance and storage at ambient temperature were not listed in the Scottish study. Those most frequently recorded for Scotland were unsafe source, inadequate cooking, cooling, thawing and reheating. The majority (120 of 147) of the Scottish outbreaks were caused by *Salmonella* spp.

Attempts are being made to reduce the level of contamination of meat and poultry but it is not going to be possible to remove all organisms. Moreover those occurring naturally, *C. perfringens* and *B. cereus*, and the human contaminant, *S. aureus*, will remain as potential causes of food poisoning. Thus prevention will require education of those involved in the preparation, processing and service of food, both on the commercial and domestic scale. The results of the analysis indicate that nearly all food poisoning could be prevented by eating food within 90 min of preparation. This is not always practical and, indeed, early preparation only leads to an outbreak of food poisoning when it is combined with other factors.

The general standard of hygiene in food premises could be improved to prevent cross-contamination by thorough cleaning and disinfection between processes, especially when both raw and cooked foods are being processed. However, the analysis shows that factors related to temperature control, i.e. storage at ambient temperature, inadequate cooling or reheating, warm holding and undercooking, are more frequently associated with outbreaks. Emphasis placed on improving this aspect would undoubtedly significantly reduce the number of outbreaks of food poisoning.

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MDPI

Review

Complementary Methods to Improve the Depuration of Bivalves: A Review

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Abstract: Bivalves are filter feeders that can accumulate and concentrate waterborne contaminants present in the water in which they live. Biotoxins, pathogenic bacteria, viruses, and heavy metals present in the aquaculture environment constitute the main hazards for human health. The most common method employed for combating waterborne pollutants in bivalves is depuration with purified seawater. Although this method is effective at increasing the microbiological quality of bivalves, in most cases, it is ineffective at eliminating other risks, such as, for example, viruses or heavy metals. Biological (bacteriocins and bacteriophages), physical (UV light, ozone, and gamma-irradiation), chemical (metallothioneins and chitosan), and other industrial processing methods have been found to be useful for eliminating some contaminants from seawater. The aim of this work was to provide a review of academic articles concerning the use of treatments complementary to conventional depuration, aiming to improve depuration process efficiency by reducing depuration times and decreasing the levels of the most difficult-to-erase contaminants. We conclude that there are different lab-tested strategies that can reduce depuration times and increase the food safety of bivalve produce, with possible short- and long-term industrial applications that could improve the competitivity of the aquaculture industry.

Keywords: depuration; bivalves; biotoxins; metallothioneins; chitosan; heavy metals

1. Introduction

The great challenge facing humanity in the coming decades is to secure food for the 9.8 billion people who are expected to inhabit the planet by around 2050 and 11.2 billion in 2100 [1]. In order to properly feed such a large population, it will be necessary to increase food production while respecting ecosystems and natural resources. The Food and Agriculture Organization of the United Nations states that aquaculture contributes to the efficient use of natural resources, food security, and economic development, with a limited and controllable impact on the environment [1]. Aquaculture is an activity that can contribute to obtaining higher yields of production by optimizing the breeding process. The world fish production reached, in 2016, a maximum of approximately 171 million tons, of which aquaculture represented 47% of the total [2]. Regarding European aquaculture, the most produced species in Europe are bivalves, such as mussels (*Mytilus* spp., including the species *M. edulis* and *M. galloprovincialis*), which represent more than 50% of the total European production [2].

A particularly important point from a food safety standpoint is that the feeding process of bivalve mollusks is by water filtration [3]. Via this process, they accumulate pathogenic bacteria, viruses, toxins, and chemical pollutants in their tissues that can pose a risk to public health [4,5]. These can then be transmitted to future consumers, with a high risk for public health [6,7].

To protect the consumer, aquaculture production areas are subject to programs of surveillance and control to avoid the presence of products carrying human pathogens [8]. The ideal procedure to obtain mollusks safely would be cultivation and harvesting in areas that are not subject to any type of contamination. However, this is unfeasible from a productive point of view because of the scarcity of these areas [7]. Consequently, depuration is a legal requirement in large countries for the marketing of fresh mollusks in order to protect consumers' health.

Currently, the most widespread method to reduce contamination is depuration, with good results in the elimination of fecal bacteria, but with variable effectiveness in the elimination of other shellfish contaminants [9]. For this reason, the behavior and accumulation of specific contaminants by filtering organisms, together with the effectiveness of depuration for their elimination, is the subject of research studies that seek to obtain safe food to protect the final consumer.

Depuration processes usually exploit the natural filtering activity of bivalve mollusks, which results in the expulsion of intestinal contents. This process reduces the probability of the transmission of infection agents to consumers through the consumption of contaminated mollusks [10]. The conventional methods used in the mollusk depuration process are chlorine, ultraviolet (UV) light, and ozone [11]. Chlorine can affect the pumping activity of mollusks, cause organoleptic changes in their meat, and cause the presence of chlorinated metabolites, such as trihalomethanes, which have carcinogenic potential [11]. In order to avoid these inconveniences, depuration plants eliminate residual chlorine from mollusks by degassing with thiosulphate and vigorous agitation of the water before introducing them into depuration facilities [12].

The use of UV light for depuration also presents disadvantages, since its efficiency depends directly on the water turbidity and flow speed, which makes this method poorly applicable for large volumes [13]. Ozone also has more variable efficacy, although like chlorine it can form potentially cancerous derivatives, such as bromates [12].

Although conventional methods are routinely successful in eliminating bacterial agents, they are rarely or not at all effective in eliminating other health risks, such as viruses, toxins, and heavy metals [10]. As a consequence, there have been some outbreaks related to mollusk consumption caused by viruses, even when the mollusks were previously purified [14]. The most common viral pathogens involved in mollusk-caused outbreaks were norovirus (higher than 80%), followed by hepatitis A virus [15]. Oysters are the most frequently implicated shellfish in viral outbreaks [15]. This increased frequency is caused by the fact that the oysters are, in many cases, consumed raw, and that it was reported that in some cases noroviruses can bind specifically to carbohydrate structure antigens in the oyster gut and can be internalized within cells of both digestive and non-digestive tissues [16,17]. Such specific ligand interactions do not only serve to bioconcentrate noroviruses in mollusks as compared to their environment but also to anchor them during the depuration processes, thus rendering depuration, which is efficacious for bacterial elimination, insufficient for the elimination of specifically bound viral particles [17].

For this reason, it is necessary to develop complementary methods that, combined with conventional depuration methods, reduce the presence of different types of contaminants in bivalves. To the best of our knowledge, this is the first review focused on complementary depuration methods that are applicable to improve or extend the efficacy of depuration of live bivalves.

2. Modification of Marine Water Employed in Depuration

Studies have shown that a rise in water temperature can favor the purification process because of an increase in the pumping and enzymatic activity by mollusks [12,18]. However, there are limits regarding the optimum purification temperature specific to each species of mollusk [19]. Thus, for different

mollusk species, there are water temperatures that, if they are exceeded, may cause negative effects in mollusks, such as a decrease in their feeding ingestion, absorption, and clearance rates, and even an increase in mollusk mortality [19]. Additionally, the use of hot marine water with different species, geographic areas, and even the season of the year showed contradictory results in terms of the elimination of both bacteria and viruses from bivalves [9,12]. In this sense, water at 25 °C showed a more effective depuration of both bacteria and viruses (poliovirus and hepatitis A virus) in oysters (*Crassostrea virginica*) compared to water depuration at 15 °C [9]. The same results were observed in clams (*Mercinaria mercinaria*) due to the more specific physiological characteristics of each species (Table 1). On the other hand, a posterior work did not find significant differences in the elimination of hepatitis A virus from mussels (*M. galloprovincialis*) depurated with marine water at 13 or 17 °C [13]. The inefficacy in reducing hepatitis A virus in mussels by increasing temperature could be explained by a negative influence of that increased temperature on the feeding processes and digestive activity of the mussels [20].

The disinfection of seawater is essential for the efficient purification of mollusks, especially in recirculation systems. One of the most used methods is disinfection by UV light, but the times and doses that are used regularly for bacterial depuration are not enough for the elimination of viruses. Thus, some enteric viruses, such as caliciviruses, show high resistance to UV treatment, requiring a UV light dose of approximately 40 mJ/cm^2 for inactivation [8]. Another highly resistant family are the adenoviruses, which require UV light at doses higher than 170 mJ/cm^2. While the degradation of the viral genome is achieved through the application of UV light on the virus, it also causes damage to the viral capsid or incapacitates the virus to infect cells [21]. Ultraviolet irradiation is effective in reducing noroviruses surrogated and hepatitis A viruses on the surface of the product but cannot inactivate viruses deep within shellfish [16]

It was shown that the amount of domoic acid (DA) produced by *Pseudo-nitzschia multiseries* and *P. australis* increases because of a defense mechanism against low levels of Fe^{3+} and Cu^{2+} that cause stress in these diatoms [22]. It was also proven that DA can be degraded by simple exposure to visible-spectrum UV light, and that this degradation is faster when Fe^{3+} is present in the seawater at up to 3 μM [18]. Therefore, in order to eliminate DA from the purification water, it is important to ensure that an adequate concentration of Fe^{3+} is dissolved in the water.

Table 1. Treatments applicable to depuration water.

Reference	Treatment	Dosage and Time	Depuration Against	Efficacy
[23]	UV light + Fe^{3+}	Continued exposure of light (full spectrum light and UV) + Fe^{3+} 0.3 mM for 22 h	Domoic acid	Degradation of 41% in the better results
[8]	UV light	Continue exposure at 44 mJ/cm^2 for 24 h	Adenovirus and norovirus	99.9% of elimination of adenovirus and norovirus after 24 h
[9]	Combinations of temperature, salinity, turbidity, pH, and the presence of algae (*Isochrysis*)	Continue exposure for 5 days	*Escherichia coli*, *Enterococcus faecalis*, coliphage MS2, Poliovirustype-1 and Hepatitis A virus	In both clams (*Crassostrea virginica*) and oysters (*Mercinaria mercinaria*), bacterial indicators were depurated faster than viral indicators

3. Depuration by Biological Methods

Bivalves can naturally contain compounds that show antibacterial, antiviral, antioxidant, and immunomodulatory effects [24]. Among them, both antimicrobial peptides and polysaccharides have received great attention in recent years [24–26].

Antimicrobial peptides are expressed by a range of animals as part of the primary defense system against pathogenic microorganisms. A large variety of small antimicrobial peptide families have been described from mollusks, mainly mussels (*Mylitus* spp.) [25]. Peptides have a small structure and provide a wide range of antimicrobial activities [27]. Antimicrobial peptides have been investigated because of their potential as new pharmaceutical substances, both for human and animal purposes [28]. In the context of intensifying aquaculture, antimicrobial peptides have been proposed as substitutes for antibiotics to prevent the selection of bacterial-resistant strains and reduce the environmental disadvantages of the use of antibiotics. However, only a few applications have been reported in extensive aquaculture, and none were aimed at being used in the depuration process [28]. Examples of works that have demonstrated the in vitro activity of antimicrobial peptides against mollusks pathogens include Defer et al. [25], whose isolated peptides from hemolymph bacteria showed inhibition in oysters (*Crassostrea gigas*) against *Bacillus megaterium* and *Micrococcus luteus,* or Ghorbanalizadeh et al. [27], whose isolated peptides from cockles (*Cerastoderma* spp.) showed inhibition against *Salmonella typhi*, *S. parathypi*, and *Staphylococcus aureus*.

In the same way, polysaccharides extracted from bivalves were shown to have a large variety of bioactivities and are usually employed in the prevention and treatment of a large variety of human diseases, including antibacterial or antiviral activities [24]. However, as in the case of antimicrobial peptides, their applications have been oriented towards other activities and not towards the purification process.

Another promising mechanism that presents interesting utilities is the use of probiotics. The term probiotics is related to bacterial species with beneficial characteristics or that can protect bivalves against infectious agents [6]. Probiotic bacteria can colonize the bivalve's digestive gland and compete for space and nutrients with potentially pathogenic bacteria [10]. Other actions include the synthesis of antimicrobial compounds and digestive enzymes that improve food conversion and nutrients' assimilation by the host and strengthen its immune system and capacity to tolerate stress [29]. The current usage of probiotics is today scarcer in mollusks than in other marine animals, such as shrimp and marine fish, but growing interest and recent advances in this field demonstrate their value [30,31]. Therefore, the application of beneficial bacteria in aquaculture can reduce or even eliminate the need for depuration of bivalves or improve the effectiveness of the technique (Table 2). In addition, these bacteria produce vitamins, enzymes, and/or essential fatty acids for the bivalve; stimulate its immune system; and can produce substances called bacteriocins with a broad spectrum of pathogen inhibitory activity [6]. Bacteriocins are proteinaceous molecules produced from bacterial strains from animals or the marine environment and have activity against other bacteria [6]. Their isolation of bacteriocin-producing bacteria was previously reported in a large variety of seafood and was applied mainly for the biocontrol of *Listeria monocytogenes* in processed foods [32]. In other cases, probiotics were able to improve the ability of mollusks to survive infection by pathogens, such as lion pan scallops (*Nodipecten subnodosus*) infected by *Vibrio alginolyticus* [30].

Table 2. Bacterial species producing bacteriocins isolated from bivalves.

Reference	Bacterial Species	Origin	Inhibition Against	Results
[34]	*Lactobacillus rhamnosus*	Oysters (*Crassostrea gigas*)	In vitro agar test of inhibition against pathogens	Good inhibition against *Vibrio alginolyticus* and *V. proteolyticus* and poor inhibition against *Edwardsinella tarda*
[6]	*Enterococcus hirae*	Mussels (*Mytilus galloprovincilais*)	In vitro agar test of inhibition against bacterial pathogens and on cellular lines for viruses	Good antibacterial activity against *Listeria monocytogenes*, and *Enterococcus faecalis*. Low antibacterial activity against *L. innocua*, good antiviral activity against Hepatitis A virus and Norovirus
[35]	Bacteriophagues	Oysters (*Crassostrea gigas*)	*V. parahaemolyticus*	Bacterial growth inhibition from 1.4×10^6 CFU/mL to 1.4×10 CFU/mL
[36]	Bacteriophagues	Cockles (*Cerastoderma edule*)	*Escherichia coli*	Reducing *E. coli* counts about 5 log CFU/g after 4-h period of depuration
[33]	*E. faecium* and *Pediococcus pentosaecus*	Oyster *(Ostrea edulis)* and clams *(Venerupis rhomboides)*	In vitro agar test of inhibition against pathogen and spoilage bacteria	Inhibition against Gram-positive bacteria, such as *L. monocytogenes*, *L. innocua*, *Staphylococcus aureus*, or *Bacillus cereus*. No inhibition against Gram-negative bacteria
[32]	Various bacterial species from genera *Bacillus*, *Paenibacillus*, *Saccharorhix*, *Pseudomonas* and *Sphingomonas*	Ark clams *(Anadara broughtoni)*	In vitro agar test of inhibition against bacteria and in vitro agar modified method for fungi and yeast	Inhibition activity of various strains isolated against Gram-positive bacteria, such as *S. aureus*, *B. subtillis*, and *E. faecium*, and even against yeast (*Candida albicans*) and molds (*Aspergillus niger* and *Fusarium oxysporum*)
[37]	Bacteriophagues	Oysters (*O. plicatula*)	*V. parahaemolyticus*	Depuration at 16 °C with bacteriophage decreased *V. parahaemolyticus* in oysters, by 2.35–2.76 log CFU/g within 36 h
[30]	*Bacillus* and *Lactobacillus* mix	Lion paw scallops (*Nodipecten subnodosus*)	*V. alginolyticus*	Increase in survival of juveniles of catarina scallop (*Argopecten ventricosus*) in 120 h
[31]	*Enterococcus faecium*	Clams (*Tapes decussatus*)	*L. monocytogenes*	In vitro inhibition activity
[25]	Peptides isolated from hemolymph bacteria (not identified)	Oysters (*C. gigas*)	*Bacillus megaterium* and *Micrococcus luteus*	In vitro inhibition activity
[27]	Antimicrobial peptides	Cockles (*Cerastoderma* spp.)	*Salmonella typhi*, *S. paratyphi* and *S. aureus*	In vitro inhibition activity for both ethanolic and methanolic solutions against *Salmonella* and *S. aureus*

CFU: Colony form units.

At the in vitro level, various bacteriocins produced by marine bacteria showed important activity against pathogenic marine bacteria. In this sense, several types of bacteria with important inhibition capacities against Gram-positive bacteria, such as *S. aureus*, *Bacillus subtillis*, or *Enterococcus faecium*, and even against yeast (*Candida albicans*), and molds (*Aspergillus niger* and *Fusarium oxysporum*) were isolated from the ark clam (*Anadara broughtoni*) [32]. Pinto et al. [33] isolated *E. faecium* and *Pediococcus pentosaecus* from oysters (*Ostrea edulis*) and clams (*Venerupis rhomboides*) with inhibition activity against Gram-positive bacteria, including *L. monocytogenes*, but no inhibition capacity against Gram-negative bacteria. Lee et al. [34] isolated *Lactobacillus rhamnosus* from oysters (*C. gigas*), which showed inhibition activity against *V. alginolyticus* and *V. proteolyticus*.

Bacteriocins can be employed in two different ways for the treatment of bivalves. The first consists of engulfing the bacteriocin-producing microorganism in the purification water at an adequate concentration to ensure that all the bivalves in the purifier meet the bacteria [6]. However, the contact with chlorinated, ozonized, or UV light-treated water that is used in conventional purification can also damage the bacterial species that produce the bacteriocins. To avoid this, the second option consists of the direct application of encapsulated bacteriocin. Although bacteriocin encapsulation is a relatively expensive process, it has already been applied in human and veterinary medicine [38]. Its combined use with the purification process was experimentally tested to reduce the amount of *V. parahaemolyticus* in oysters [35,37]. The results show a reduction of nearly 6 log CFU/g in *V. parahaemolyticus* in oysters (*O. plicatula*) after a combined purification process with phages for 72 h.

Another interesting way to improve the depuration process in mollusks is the use of bacteriophages, which were employed in cockles (*Cerastoderma edule*) and eliminated concentrations of about 5 log CFU/g of *Escherichia coli* after 4 h of depuration. These methods showed a great acceleration of the process with respect to conventional depuration methods. In addition, the phages employed in the process do not remain in the final product, since they are destroyed after a period of exposure to ultraviolet light from solar radiation, which improves the product's safety [36].

4. Depuration by Physical Methods

Different physical methods tested at an experimental level showed effectiveness in the elimination or reduction of pathogenic agents in bivalves. Some examples are the application of various temperature treatments [39,40], X-ray irradiation [41], γ-irradiation [42,43], ozone [44–47], and the application of high hydrostatic pressure (HHP) alone [48] or combined with bacteriophages [48]. The effectiveness of the use of the combination between several of the previous techniques, such as the high hydrostatic pressures combined with a moderate heating [49], or the high hydrostatic pressures combined with the use of bactericidal phages [48], was also demonstrated (Table 3). However, the bivalves die during these processing techniques, modifying their nutritional and organoleptic characteristics; because of this, the application of these techniques is only useful on products that are going to be commercialized or transformed and never on fresh products [36].

Table 3. Complementary depuration methods using physical procedures.

Reference	Treatment	Dosage and Time	Bivalve Species	Inhibition Against (Efficacy)
[48]	High hydrostatic pressure (HHP)	550 MPa for 5 min	Blue mussels (*Mytilus edulis*)	*Shigella flexneri* and *Vibrio cholerae* (complete elimination from 3.8 log CFU/g)
[44]	Ozonation	360 mg ozone/h for 3 days	Mussels (*M. galloprovincialis*)	Diarrheic shellfish poisoning (DSP) reduced toxicity in mouse after 3 days
[39]	Flash freezing and frozen	Flash freezing, followed by storage at −21 +/−2 °C for 5 months	Pacific oysters (*Crassostrea gigas*)	*Vibrio parahaemolyticus* and *Vibrio vulnificus* (3.52-fold log MPN/g)
[42]	Ozonation	15 mg/kg for 6 h	Mussels (*M. galloprovincialis*)	Okadaic acid (21%–66% reduction)
[42]	γ-irradiation	6 kGy	Mussels (*M. galloprovincialis*)	Okadaic acid (10%–41% reduction)
[41]	X-Ray	1–5 kGy	Oysters (*Crassostrea virginica*)	*V. parahaemolyticus* (4-fold log CFU/g)
[43]	γ-irradiation	6, 12, and 24 kGy	Mussels (*M. edulis*)	Domoic acid (40%–100%), azaspirazids (15%–50%), Okadaic acid (0%–30%), pectenotoxin (30%–75%), yesotoxins (0%–15%), depending of the dose
[40]	Refrigeration	Depuration at controlled temperature between 7–15 °C for 5 days	Oysters (*C. gigas*)	*V. parahaemolyticus* (3-fold log MPN/g)
[46]	Ozonation under different pH	1.24 V	Chemical analyses and mice bioassay	Ozone was more effective under acidic conditions and combined with hydrogen peroxide than alone conditions (2.07 V)
[47]	Ozonation	25 mg ozone/L for 30 seg	HPLC and fish (*Cyprinodon variegatus*) bioassay	*Gymnodinium breve* toxins showed 3-log CFU cycle reduction in the total toxin recovered after 10 min (135 mg/L) of ozone exposure
[49]	Temperature combined by high hydrostatic pressure	HHP at ≥275 MPa for 2 min followed by heat treatment at 45 °C for 20 min; HHP at ≥200 Mpa for 2 min followed by heat treatment at 50 °C for 15 min	Oysters (*C. virginica*)	*V. parahaemolyticus* and *V. vulnificus* (3-fold log MPN/g)

CFU: Colony form units; HHP: high hydrostatic pressure; Mpa: Megapascal; MPN: Most probable number.

Physical methods based on the application of special temperatures are aimed to decrease microbiological risks (*Vibrio* spp.) and can consist in flash freezing [39], water refrigeration [40], or thermal treatment combined to HHP [49]. Refrigeration is the usual method to maintain and transport live bivalves, and the use of slurry ice was previously demonstrated to improve their microbial and sensory quality [50].

Food irradiation is recognized as an effective technology for the elimination of pathogens that contaminate crude food [51,52], thus there are very few studies on the application of this treatment for the depuration of bivalves [53]. This treatment is only regulated in some EU countries, where the use of 3 kGy radiation is allowed for the treatment of fish and shellfish, which can be increased to 5 kGy for unpeeled and/or decapitated prawns [40]. In addition, the European Food Safety Authority (EFSA) [54] also intends to set a limit of 5 kGy for the irradiation of both fresh and frozen seafood products.

A previous work demonstrated that there is a significant reduction (10%–41%) in okadaic acid (OA) levels in mussels after the application of irradiation [42]. In addition, in their study, they also evaluated negative (non-toxic) samples to verify that there was no formation of acute toxicity-producing compounds; samples remained negative after treatment. However, they did not rule out the possibility that this treatment may form compounds that produce long-term toxicity or which have carcinogenic effects, such as 2-alkylcyclobutanones, which are radiolytic derivatives of triglycerides that are contained exclusively in irradiated foods and which have been experimentally shown to promote colon cancer [55].

Studies regarding the application of ozone to lipophilic toxins and diarrheic shellfish poisoning (DSP) toxins are limited [44,45]. Additionally, these studies only assess the effectiveness of ozone treatment on the water in which the mollusks are kept, not on the final product, since they can only be applied before harvesting while mollusks are alive.

To evaluate the efficacy of ozone treatment applied to the final product in the elimination of DSP toxins, Louppis et al. [42] ozonized samples of homogenized mussels and whole unshelled mussels inside a refrigerator at 4 °C at a dose of 15 mg/kg for 6 h. The results obtained show that this treatment is effective in reducing the amount of OA and its derivatives independent of the initial degree of contamination. Thus, in the worst case, this treatment provided a 21% reduction in the content of this toxin in mussels (*M. galloprovincialis*), which experimentally was enough to change a product that could not be marketed to one that complies with legal limits. Thus, it should be noted that a greater reduction in OA was obtained in homogenized mussel samples than in whole mussel samples. This was attributed to the fact that the treated mussel tissue was from the hepatopancreas, which has a much higher lipid content than whole mussel tissue, a factor that interferes with the capacity of ozone to interact with the toxin, because OA is lipophilic [42].

A possible explanation for the effectiveness of treatment with ozone on the OA content of mussels could be the interaction of the gas with the double bonds of the OA molecule [56]. It is known that ozone attacks the double bonds of organic compounds [57] and that the OA molecule contains several double bonds that could be a potential target for ozone. Therefore, the alteration in the OA structure that occurs after the treatment could be caused by the reduction in its concentration, because the detection procedures would not recognize the altered molecule. Consequently, it is necessary to continue investigating the mechanism of action of ozone to confirm its effectiveness and potential applications in industry.

5. Depuration by Chemical Methods

Conventional purification processes show slow elimination of certain undesirable compounds, such as marine toxins and heavy metals. Bivalves accumulate heavy metals slowly throughout their life, and their elimination is tremendously slow when treated only with clean seawater [58]. The heaviest heavy metal traditionally known to be dangerous to human health is Hg^{2+}, with greater importance in pregnant women and children [59,60]. However, other targets, such as Cd^{2+}, also represent an important risk, as a consequence of their solubility and because they are capable of producing problems

in the immune and reproductive systems and have a potentially teratogenic effect [61]. The fact that this element is more soluble in acidic media implies that its presence in fishery products will be greater in the future because the oceans are undergoing an acidification process as a consequence of climate change [61].

In addition to fighting microbiological risks by co-purification, it is necessary to develop systems capable of reducing chemical agents in bivalves. One of the strategies proposed for this purpose is the use of chelating agents, which join heavy metals or other toxins, reducing their availability or facilitating their elimination [59]. Chelating agents currently present great potential for use in the food and health fields due to their antimicrobial, immunomodulatory, antitumor, and antioxidant effects, as well as their capacity to chelate and diminish heavy metal availability [62]. The specific usages of chelating agents in bivalves depuration can be shown in Table 4.

Among the chelating substances of interest for their application in aquaculture, metallothioneins (MTs), which are naturally produced by bivalves [58], are highlighted. MTs are proteins rich in cysteine and have a low molecular weight. They cover a wide range of organisms and show a remarkable affinity for targets, such as Zn^{2+}, Cd^{2+}, and Ca^{2+} [62,63]. The MTs contained in bivalves, such as mussels (*M. edulis*), play an important role in the transport and storage of heavy metals, but they also provide them with a certain protective function (detoxification effect) against excessive amounts of non-essential metals, such as in the case of Cd^{2+}, Ag^{2+}, or Hg^{2+} [58].

Recent work showed that the addition of MTs obtained from fishing subproducts achieved a reduction, through the formation of complexes containing Cd^{2+} in mussels (*M. edulis*) in the gills, mantle, and viscera, of around 30%, 40%, and 25%, respectively, after a 15-day exposure [64]. The same authors in a further work demonstrated a better elimination (about 50%) of Cd^{2+} from mussels (*M. edulis*) when employing MTs protein hydrolysate complexed to Fe^{2+} [64].

In a previous study, it was demonstrated that using MTs at the same time as traditional methods of depuration failed in reducing the concentration of Cd^{2+} significantly because its depuration kinetics are extremely slow [65]. Baudrimont et al. [66], in a previous work, also obtained good depuration results for Cd^{2+}, Al^{2+}, and Hg^{2+} using MTs in Asian clams (*Corbicula fluminea*).

Table 4. Chelating agents used in bivalve chemical hazards' depuration.

Reference	Chelating Agent	Dosage and Time	Bivalve Species	Inhibition Against (Efficacy)
[66]	Metallothioenins (MTs)	ND	Asiatic clams (*Corbicula fluminea*)	Cd^{2+} sequestered by the MTs fraction represented 40% of the total Cd^{2+} bioaccumulated in the soft body of the mollusks, compared with 4%–9% for total accumulated Zn^{2+}
[67]	Chitosan oligosaccharide + Ca^{2+} (COS-Ca)	Different doses ranging 1.75–8.75 mg/L for 6 days	Scallops (*ChlamysFerrari*)	COS-Ca reduced Cd^{2+} content of the scallops, with highest depuration rate (47%) observed on day 3. Additionally, increased Ca^{2+} content (73.9%) on day 6, and did not significantly affected Zn^{2+} content
[69]	Chitosan, *Chlorella* and Chitosan + *Chlorella*	8×10^3 cells/mL *Chlorella*, 0.05 g/L chitosan, and combination of both	Oysters (*Ostrea rivularis*)	Toxicity caused by paralytic shellfish poisoning decreased from 9.07 mouse units (MUs) to 1.41 MUs using chitosan and 0.12 mouse units using chitosan plus *Chlorella*
[58]	MTs (protein hydrolysate-Fe^{2+})	40 mg/L protein hydrolysate-Fe^{2+} for 15 days	Blue mussels (*Mytilus edulis*)	Cd^{2+} concentration in blue mussel decreased from 46.1 to 23.3 μg/g
[58]	MTs (hydrolysis peptide–metal element complexes (Fe^{2+}, Zn^{2+}, Ca^{2+}, or Hg^{2+})	Different concentrations of MTs (5, 10, 15, and 20 mg/L) for 8 days	Blue mussels (*M. edulis*)	Cd^{2+} decreased in the range 25%–40% after exposure to 20 mg/L of hydrolysis peptide–metal element complexed to Fe^{2+}, Zn^{2+}, and Ca^{2+} No significant decrease was found for hydrolysis peptide–metal element complexed to Hg^{2+}
[68]	Combinations between chitosan, ozone and hydrodynamic treatment	1.5 mg/L ozone, 0.5 mg/L chitosan and 1.3 m/s hydrodynamic treatment for 60 min	Green mussels (*Perna Viridis* L.) and blood cockles (*Anadara granosa* L.)	The most effective combination was chitosan-ozone, achieving a Hg^{2+} depuration of 96.5% in green mussels and 87% in blood cockles

Other natural compounds, such as chitosan, can also achieve this chelating action of heavy metals. Chitosan is a long-chain polysaccharide obtained by the distillation of chitin from crustacean shells [67]. More recently, Widiah Ningrum et al. [68] developed a system to reduce the level of Hg^{2+} in green mussels (*Perna viridis* L.) and in cockles (*Anadara granosa* L.). Chitosan is only soluble under acid conditions; for this reason, it is essential to use it in a way that favors its solubility [67]. Thus, chitosan can be dissolved in 5% acetic acid to form a gel. This gel with chitosan is administered by pumping it, together with ozonized seawater (at a concentration of 1.5 mg/L ozone and 0.5 mg/L chitosan), at a rate of 1.3 m/s for 1 day. This achieved a reduction in the Hg^{2+} content of more than 90% in mussels (*Perna viridis* L.), and of around 85% in the case of cockles (*A. granosa* L.) [68].

In another work [69], chitosan was also used for the experimental purification of oysters (*Ostrea rivularis*) over 7 days. During this period, it was possible to reduce the quantity of paralytic shellfish-poisoning (PSP) toxins in oysters by more than 60% when using chitosan, and by more than 85% when chitosan was administered in combination with *Chlorella* microalgae.

In the same way, Huang et al. [67] employed Chinese scallops (*Chlamys ferrari*) to study the purification process over 7 days with different concentrations of chitosan combined with calcium to facilitate its solubility. These researchers measured the concentrations of Cd^{2+}, Ca^{2+}, and Zn^{2+}, demonstrating that, by means of this system, the concentrations of Cd^{2+} were reduced by 18% while those of Ca^{2+} and Zn^{2+} were not reduced by significant amounts. This uncontrolled reduction of Cd^{2+} implies an increase in the purification speed with respect to conventional methods [67].

6. Methodologies for Decontamination during Industrial Processing

Contamination by DSP toxins in mussels and by amnesic shellfish poisoning (ASP) in scallops are very important concerns in European aquaculture. When the mussels are contaminated with OA, natural detoxification takes several weeks; after that, the phenomenon of proliferation of toxic algae occurs [70], which causes great economic losses in the aquaculture sector. The problem is greater in the case of scallops (*Pecten maximus*), because their metabolism produces extremely slow detoxification processes and they can remain toxic for several months [71].

Several treatments have been studied to evaluate the reduction of toxicity by ASP in scallops, individually and in combination, including evisceration (extirpation of the digestive system and hepatopancreas), thermal treatment carried out on the product after removing the meat from the shell, applying a series of cooking and washing methods that finished off each sterilization in an autoclave at 116 °C for 54 min, and freezing to −20 °C. All methods resulted in an insignificant reduction in toxicity; the only method that was able to reduce the values to the legal limit was ablation of the hepatopancreas, achieving the almost complete elimination of the toxin [45].

The application of a thermal process was able to reduce the levels of PSP toxin significantly, especially in mussels (*M. edulis*), and even in clams (*Rudipates decussatus*) and cockles (*Cerastoderma edule*). The process consisted of a succession of washing and heating phases; the maximum peak temperature was 98 °C for 9 min followed by autoclaving at 116 °C for 54 min [45]. At the experimental level, it was also proven that the use of alkaline solutions followed by cooking and washing reduces the levels of this toxin [45].

Regarding DSP toxicity in mussels (*M. edulis*), the effect of freezing, ozonization, thermal treatment, and thermal treatment with additives was analyzed; diverse results were obtained for the elimination of OA. Detoxification for a month or more did not manage to modify the levels of OA [45]. There are references to the possibility of reducing its levels by using supercritical CO_2, but the product obtained using this technique is not acceptable from a commercial point of view [72]. Another mechanism studied is the addition of N-acetylcysteine, a precursor of glutathione that would increase the speed of the detoxification mechanisms [73].

The maintenance of mussels (*M. edulis*) in ozonized sea water with a redox potential >450 mV for 24 h was not able to eliminate the DSP toxin from its interior, but it was able to cause a significant

increase in the proportion of OA/total DSP [45], which shows that the oxidative power of ozone changes the profile of toxins in mussels.

Thermal treatment of mussels is not effective for decreasing the content of DSP toxin. However, the content of ASP and PSP toxins reduced significantly but without reaching values under the legal limits [45]. To achieve effective decontamination, it is necessary to combine thermal treatment with other methods, such as evisceration and freezing for ASP, and freezing for PSP.

There are several hypotheses regarding the difficulty of eliminating DSP toxin; in the scientific literature, there are references to OA being stored in different compartments within the organism presenting different isoforms [45]. Thus, OA shows different kinetics of detoxification and interconnection between its isoforms over time [74]. Furthermore, it has also been proposed that within these compartments, the toxins remain stored in inactive liposomes [75], which complicates the access to treatments, even though the main place of storage is the hepatopancreas.

7. Conclusions

There is no single method that can be successfully applied to all species of bivalves and protects against all sanitary risks. Some of the mentioned methods showed promising results at the experimental level, even for the elimination of very persistent pollutants. However, they have not yet been tested at the commercial level. For this reason, it is fundamental to transfer the knowledge to the production sector by promoting links between research and industry. The final aim is to improve the competitiveness of bivalve aquaculture, a sector of great current importance and which will be even more important in the future. In addition, these new methods in combination with traditional purification results in an increase in food security for the population.

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Final Report: Evaluating the effectiveness of depuration in removing norovirus from oysters

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Executive Summary

Pollution of coastal waters with non-treated, or partially treated, human effluent can result in contamination of bivalve molluscan shellfish with microbial pathogens, including human enteric viruses. Norovirus (NoV) is now considered to be the main cause of human gastroenteritis worldwide, and is the most common viral pathogen associated with illness from shellfish consumption. Oysters are the most common shellfish species implicated in NoV outbreaks and recent studies in the UK have suggested that a significant proportion of oyster production areas contain NoV RNA.

The reduction or elimination of microbial contaminants from oysters via the process of filter feeding can occur in either the natural environment in unpolluted seawater (relaying) or in land based facilities (depuration). Depuration is an effective post-harvest treatment for the removal of most bacterial species from oysters (with the notable exception of *Vibrio* spp.) and has successfully reduced illness outbreaks of typhoid and cholera. However, the efficiency of depuration for the removal of NoV is questionable. This study was therefore commissioned to evaluate the efficacy of depuration in removing NoV from oysters through a review of the literature (Part 1), and to undertake pilot laboratory experiments to investigate a novel depuration approach (Part 2).

The literature review has identified at least 17 published articles which report illness outbreaks of NoV and hepatitis A virus (HAV) from the consumption of oysters that were subjected to depuration. Reported concentrations of NoV in oysters post depuration were between 10^2 and 10^3 genome copies/g oyster tissue, far in excess of the infectious dose which is estimated to be as low as 10 viral genome copies.

Optimising environmental conditions such as temperature and salinity to suit the physiology of oysters results in increased depuration rates for bacteria and some viral indicator species. This relates to maximising oyster clearance, filtration and digestion rates. However, these changes only result in small or no improvements in viral depuration rates for NoV and HAV, suggesting a special relationship between NoV and oysters. Indeed, genogroup I (GI) and II (GII) NoV bind to histo-blood group (HBGA) A-like ligands in oyster digestive tissues and GII NoV binds to sialic acid (SA) residues in the gills; NoV binding to these ligands is hypothesised to facilitate accumulation. Additionally, NoV and HAV show high acid resistance which may also contribute to prolonged persistence within the acidic environment of oyster haemocytes.

The differences in the way in which NoV interacts with oysters compared to other surrogate viruses which can be cultured (e.g. feline calicivirus, murine norovirus) is highlighted by the findings of this review, which shows that NoV and HAV are retained for much longer periods in oysters than surrogate viruses. For NoV, 50% of published reduction experiments (n=16) showed no reduction in levels during depuration, and for studies in which reductions did occur it took between 9 and 45.5 days for a 1 log reduction. For HAV, the estimated days to achieve 1 log reduction was between 7 and 16.1 days, with two of the five experiments showing no loss of HAV. These timeframes are clearly much longer than those used routinely for depuration in the UK (around 42 h). The culturable surrogate viruses are more rapidly depurated than NoV and HAV under a variety of depuration conditions; a comparison of the days to achieve 1 log reduction shows that the mean number of days to reduce NoV and HAV is 19 and 12 respectively, whereas for surrogate viruses the mean time to achieve 1 log reduction is 7.5 days. Given the special relationship between NoV and oysters, it is unlikely that optimising the physiology of oysters through parameters such as salinity, temperature, and presence/absence of food will result in significant reductions, as demonstrated by studies to date.

Given that depuration does not reduce NoV to levels below an infectious dose and illness outbreaks have occurred from consumption of depurated oysters, depuration is not an effective post-harvest control for NoV in oysters at this time. On the other hand, relaying has been more successful, with NoV reduced to around the LoD of the test method when oysters are placed in clean open seawater for around four weeks, and no illnesses have been reported to be associated with relayed products.

This literature review has identified a number of data gaps and uncertainties, including:

- There is no direct information on the infectivity of NoV following depuration and relaying, however some information on infectivity can be inferred from studies with HAV for which a culture method exists, and from illness outbreaks that have occurred following depuration;
- The quantitative test methods for NoV used in some historical depuration studies may not have been appropriate, due to a lack of quantitative viral standards and lack of rigour in the sampling plans used;
- The biological basis of why HAV persists in oysters for long periods is unknown, further work on specific interactions between HAV and oysters may assist in elucidating this;
- For most illness outbreaks involving depurated oysters, the depuration conditions used are not known/stated;
- Few depuration studies have sought to optimise a combination of conditions;
- The relative rates of depuration of NoV from Pacific and Native oysters is unknown; and
- The depuration rates of naturally contaminated oysters vs. lab contaminated oysters has not been investigated.

Thus, it is suggested that the following topics be given high priority when considering further research and work in this area:

- A major focus should be placed on improvements in coastal water quality. Further collaboration between UK water companies, Local Authorities and industry should be prioritised in order to develop cohesive and practical strategies to achieve this goal.
- Collaborative research to investigate the infectivity of NoV in oysters during depuration and relaying.
- Improving understanding of the virus-oyster relationship and specifically the binding interactions.
- Investigations into post-harvest interventions that aim to disrupt the specific binding of NoV to oysters.

Depuration approaches which include a step to exploit/disrupt the specific linkage between NoV and HBGAs in oysters may enhance the reduction of NoV. As part of this project a pilot study (Part 2) was undertaken to investigate if such an approach holds promise. The idea was to test different compounds which were selected on the basis of their activity against HBGAs, with the hypothesis that the treatment would destroy the ligands and lead to the release of NoV particles inside the oyster tissues during depuration.

The efficacy of eight different compounds in reducing levels of NoV in oysters were evaluated using two experimental approaches. Firstly, ten depuration trials were conducted in which oysters contaminated with NoV were dipped in one of the selected compounds and then subjected to depuration; and secondly an *in vitro* approach was used to evaluate compound efficacy, this involved treating the digestive tissue and gills of oysters that had accumulated NoV with different compounds in cell culture plates.

The results of the dipping and *in vitro* experiments showed that two compounds, proteinase K and papain, have promise in further reducing NoV concentrations during depuration. The development of the *in vitro* test was valuable and allowed more rapid screening of potentially effective compounds.

While the pilot studies show promise, further experiments are recommended to confirm the potential of these compounds to enhance depuration:

1. Quantitation by real time RT-PCR was not precise enough to discriminate levels within a log; further work using a more precise method such as digital PCR would assist in further evaluating efficacy of the compounds.
2. The effect of proteinase K and papain were evaluated using grossly contaminated oysters; the work should be repeated using oysters contaminated at a lower level (such as those naturally contaminated in the environment), and for accidental contamination events in which oyster exposure to NoV is short (i.e. for less than an hour).
3. Lastly, the reduction effect may be enhanced further by trialling options to deliver the compounds more directly to the sites of interest within oysters. In this regard, microencapsulation of the compounds of interest may be an interesting avenue to pursue.

Glossary

CEFAS	Centre for Environment, Fisheries and Aquaculture Science
dPCR	Digital Polymerase Chain Reaction
DALYs	Disability Adjusted Life Years
DT	Digestive tissue
EU	European Union
EC	European Commission
FCV	Feline calicivirus
FRNA	F-specific RNA
FSA	Food Standards Agency
GI	Genogroup I
GII	Genogroup II
GIV	Genogroup IV
GMT	Geometric mean titre
HAV	Hepatitis A virus
HBGA	Histo blood group antigen
ID_{50}	Infectious dose
ISO	International Organisation for Standardisation
LA	Local authority
LoD	Limit of detection
LoQ	Limit of quantitation
MgV	Mengo virus
MNV	Murine norovirus
NoV	Norovirus
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PFU	Plaque forming units
PK	Proteinase k
PV	Poliovirus
RASFF	EU Rapid Alert System for Food and Feed
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
RT-qPCR	Real time quantitative reverse transcriptase polymerase chain reaction
SA	Sialic acid
ST	Supernatant component
TV	Tulane virus
UK	United Kingdom
UV	Ultra violet
VLP	Virus-like-particles
WHO	World Health Organization

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Part One: Literature Review of the Effectiveness of Depuration in Removing Norovirus from Oysters

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1 Introduction

1.1 Background

Pollution of coastal waters with non-treated, or partially treated, human effluent can result in contamination of bivalve molluscan shellfish with a variety of microbial pathogens, including human enteric viruses. Human enteric viruses replicate in the human alimentary tract with large quantities of virus shed in the faeces (Atmar et al., 2008; EFSA, 2011). An important enteric virus, norovirus (NoV), is the predominant cause of human gastroenteritis. Recent estimates from the WHO on the global burden of foodborne diseases suggest that the most frequent causes of foodborne illness were diarrhoeal disease agents, particularly NoV and *Campylobacter*; NoV gastroenteritis was estimated to contribute 7.6% of the total DALYs (Disability Adjusted Life Years) (Anon, 2015a).

Globally, NoV is the most common viral pathogen associated with illness from shellfish consumption. Bellou et al. (2013) found that 83.7% of shellfish borne viral illness outbreaks identified (n=359) involved NoV, and 12.8% involved HAV. Bivalve shellfish may become contaminated with NoV and HAV through the process of filter feeding, in which large quantities of seawater and associated particulate matter, including any enteric viruses present, are ingested and localised in the digestive tract. While all bivalve shellfish are susceptible to contamination, oysters are more frequently implicated in illness outbreaks than other shellfish species: Bellou et al. (2013) conducted a systematic review to investigate shellfish borne viral outbreaks and found that the most common type of shellfish involved in outbreaks were oysters (58.4% of outbreaks). Clams were responsible for a reported 22.6% and mussels for 0.5% of outbreaks (Bellou et al., 2013).

There are several possible reasons why oysters appear to play a more dominant role in the transmission of NoV compared to other shellfish species, including:

1. The mode of consumption (primarily raw);
2. The close proximity of inter-tidal oyster production areas to sources of human effluent;
3. The specific retention of NoV in oysters via binding to ligands that are present within the oyster tissues (Le Guyader et al., 2006a; Le Guyader et al., 2012); and
4. Relatively slow inactivation and elimination of NoV from oyster tissues (McLeod et al., 2009a, 2009b; Richards et al., 2010).

Oysters are an important commodity in the UK, with approximately 1300 tonnes produced annually and considerable potential for industry expansion. Two species of oyster are commercially produced, the Pacific oyster (*Crassostrea gigas*) (91% of oyster production) and the Native oyster (*Ostrea edulis*). Figure 1.1 provides an overview of oyster production in the UK between 2010 and 2013 (the latest available production statistics). In accordance with EU law, bivalve shellfish production areas in the UK are classified as A, B or C, based on the presence and levels of the faecal indicator bacteria, *E. coli*. Shellfish harvested from areas that are classified as B must be subjected to depuration, cooking or relaying. In England and Wales, in 2014/15 there were 111 areas classified for the production of oysters, of which 101 were class B. Over the same period in Scotland, 33 areas were classified for the production of oysters, of which 20 were class A/B, and 13 were class A. Northern Ireland had 12 areas classified for oyster production, of which 7 were class B. Given (1) the prevalence of class B areas in the UK (78% are class B in particular seasons), (2) the requirement to either depurate, cook or relay shellfish from class B areas, and (3) the fact that cooked oysters are not normally considered a

desirable product by consumers, there is a heavy reliance by the UK oyster industry on depuration as a process control (as opposed to cooking).

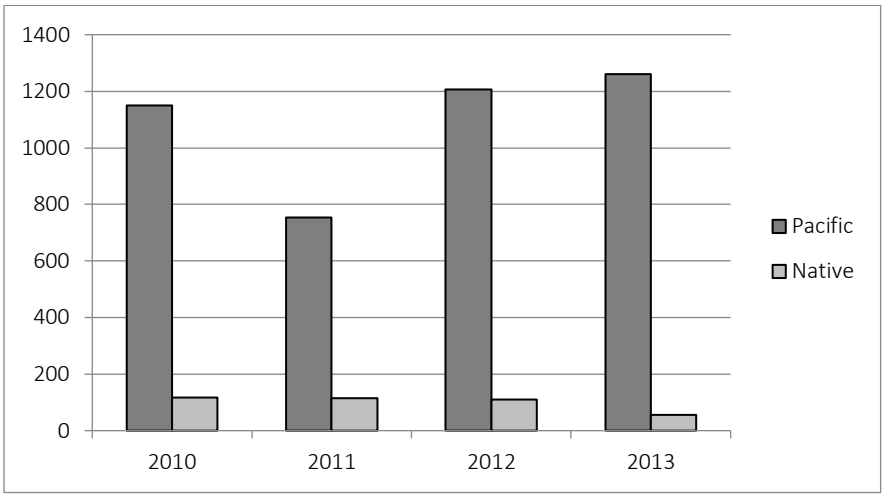

Figure 1.1. Oyster production (tonnes) in the United Kingdom (England, Wales, Scotland and Northern Ireland) between 2010 and 2013.

While it is clear that depuration is a highly effective post-harvest treatment process for removing *E. coli*, the efficiency of depuration for the removal of NoV is questionable, with several studies suggesting low reduction rates (McLeod et al., 2009a; Schwab et al., 1998; Ueki et al., 2007), and some cases of NoV related illness from oysters that had been depurated (Gallimore et al., 2005; Grohmann et al., 1981; Le Guyader et al., 2010; Stafford et al., 1997).

The illness outbreaks have prompted a variety of scientific investigations into NoV contamination of oysters, including a study to evaluate the prevalence of NoV in UK oyster production areas. The study suggested that a significant proportion of the oyster production areas contained NoV RNA, with each of the 39 production areas tested giving at least one NoV positive result (using real time Polymerase Chain Reaction (PCR) to detect the viral RNA) during the study (Lowther et al., 2012). The recent findings of high prevalence of NoV RNA, the reported illness outbreaks, and the high reliance by industry on depuration as an end product control, have together led to the prioritisation of research to evaluate the effectiveness of depuration for NoV reduction in oysters (Anon, 2012) (Food Standards Agency Conference, 2013). Elimination of NoV from oysters via depuration is thus the subject of this review.

The reduction or elimination of microbial contaminants from oysters via the process of filter feeding can occur in either the natural environment in unpolluted seawater or in land based facilities. For the purposes of this review, elimination in a natural setting is referred to as relaying, and elimination in tanks is referred to as depuration (also frequently described as 'purification' in the literature).

1.2 Aims of the review

The overall objective of this review is to evaluate the effectiveness of depuration in removing NoV from oysters. Minor additional aims of the review include:

1. To evaluate the effectiveness of relaying in removing NoV from oysters;
2. To summarise current depuration practices in the UK and overseas;
3. To review the mechanism by which NoV is bound and retained in oysters; and
4. To identify new potential approaches for viral depuration.

1.3 Scope of the review

The review involves the appraisal of literature with respect to the efficiency of depuration in removing NoV from oysters, the following study types were considered in the review:

- Depuration studies of commercially produced oyster species (including Pacific and Native oysters - *Crassostrea gigas* and *Ostrea edulis*);
- Depuration of both GI and GII NoV strains and HAV;
- Depuration of viral surrogates for NoV e.g. F+ bacteriophage;
- NoV illness outbreaks from oysters which had been depurated; and
- The binding mechanism of NoV to oysters.

The following subjects were not considered in the review:

- Depuration studies of non-oyster shellfish species;
- Depuration studies of non-related viruses and bacteria (e.g. poliovirus, *E. coli*); and
- The binding mechanism of non-related viruses and bacteria to oysters or other shellfish.

2 Approach to the Review

2.1 Literature review

Literature searches were undertaken to collate information on the following subjects:

- Illness outbreaks caused by NoV in depurated oysters;
- Persistence of NoV in oysters following depuration and/or relaying; and
- The mechanism by which NoV is bound and retained by oysters.

Depuration studies of non-oyster shellfish species and bacteria were not considered in this review. Information on the depuration of other viruses, particularly hepatitis A virus (HAV) and so called indicator or surrogate viruses were also collated.

Literature searches began with a structured electronic search using the Google Scholar and PubMed search engines. Electronic literature searches commenced with the following key words:

1. oyster AND outbreaks AND depuration AND (norovirus OR Norwalk virus)
2. (norovirus OR Norwalk virus) AND oyster AND (persistence OR relaying OR depuration)
3. (norovirus OR Norwalk virus) AND oyster AND (localisation OR binding)

For the first literature search above, the titles and abstracts of the first 300 citations identified and sorted by Google Scholar (n=1660) were reviewed for relevance. All abstracts of the citations identified using PubMed (n=119) were reviewed. Articles were included in the review if it was clear that oysters implicated in outbreaks had been subjected to depuration; only 15 articles met this criterion. The outbreaks identified spanned the period 1979 to 2012. A further 30 articles were identified that described outbreaks attributed to oyster consumption in which the oysters either had not been depurated, or it was not stated if depuration occurred, and were thus excluded. The following information was collated and tabulated from the identified publications:

- The number of cases in the outbreak
- Year of the outbreak
- Country of the outbreak
- Country of the oyster origin
- Information regarding an epidemiological link between outbreak and oysters
- Analytical confirmation of NoV in human faeces and oysters
- Levels of NoV present in oysters post depuration.

For the second literature search noted above, the titles and abstracts of the first 300 citations identified and sorted by Google Scholar (n=1270) were reviewed for relevance. All abstracts of the citations identified using PubMed (n=29) were reviewed. Of the identified publications, 14 peer reviewed research articles contained quantitative data on the reduction of NoV, HAV and viral surrogates in oysters during depuration and relaying, and thus, the data were included in the review. To compare the reduction rate of NoV, HAV and surrogate viruses across different studies, viral levels in oysters were determined for each study from either raw data reported in publications, or estimated from graphs using WebPlotDigitizer (http://arohatgi.info/WebPlotDigitizer/). Similar to other authors (Choi and Kingsley, 2016; Loisy et al., 2005; Love et al., 2010), it was assumed that viral loss was exponential and a linear regression model was fitted to the estimated $\log_{10}$ transformed viral concentrations to assess the reduction in viruses over time (Excel, 2016, Microsoft®). The models were then used to predict an estimated average single log reduction time for viruses within oyster tissues for each published study (Tables 1.4, 1.5 and 1.6).

For the third literature search on localisation of NoV, the titles and abstracts of the first 300 citations identified and sorted by Google Scholar (n=1280) were reviewed for relevance. All abstracts of the citations identified using PubMed (n=12) were reviewed.

Non-English language studies were not included. Additional papers were accessed using the reference list of reviewed publications.

2.2 Critical appraisal process

A critical appraisal of the most relevant papers included in the review was undertaken. The appraisal process used was similar to that undertaken in a recent literature review by McLeod (2014). This involved the following steps:

1. Papers were initially filtered to identify those that are most relevant to the objectives of this study. The criteria used to identify papers of highest relevance included:
    - Studies in which Pacific and/or Native oysters were contaminated with NoV (either naturally or through bioaccumulation) and subjected to depuration or relaying and the concentrations of NoV measured before and after the cleansing process.
    - Reports of illness outbreaks related to NoV in oysters that had been subjected to depuration and/or relaying.
    - Studies that significantly influenced the outcomes of the literature review conclusions.

2. Studies of high relevance (as identified in 1 above) were evaluated using the following questions:
    - Were appropriate analytical test methodologies used for NoV (i.e. the ISO standard method or equivalent)?
    - Were depuration parameters noted in the study (e.g. temperature, salinity, time, disinfection approach, flow rate, loading density)?
    - Did the study design, data and statistical treatment support the conclusions?
    - For uptake and depuration studies:
        - Were oysters maintained in appropriate conditions to ensure they were alive and functioning optimally?
        - Was the number of sampling occasions and/or oyster samples analysed sufficient to support conclusions regarding relative elimination efficiency for NoV?
    - For reports on illness outbreaks related to NoV in oysters:
        - Does the epidemiology evidence presented strongly implicate oysters as the vector (i.e. were the epidemiology investigations analytical and have statistics presented)?
        - Were oysters tested for the presence of NoV?

3. The questions above were evaluated for each of the high relevance papers, and a score of 0 (no), 1 (acceptable/generally) or 2 (yes) was allocated for each question. A total score was calculated for each paper, thus high scoring papers are suggestive of robust results and conclusions (a maximum score of 10 is possible). The results of the critical appraisal for each relevant paper are presented in Appendix 1.

2.3 International depuration practices: survey approach

A key objective of this project is to evaluate the efficacy of the depuration process in reducing the levels of NoV in oysters. To fulfil this objective it is ideal to have robust information on two different subjects: (a) the way in which depuration is performed currently by the oyster industry (what the current process entails), and (b) the results of NoV reduction studies on oysters that have been depurated. If robust depuration studies on NoV have been conducted using a depuration process that is broadly similar to commercial processes currently used in the UK, this should enable a desktop evaluation of the efficacy of the process.

Thus, to gain insight into the way in which oysters are currently depurated in the UK and overseas, two questionnaires (one for depuration operators in the UK, and the other for overseas industry members) were prepared to seek information, particularly focused on the process parameters used during depuration, such as seawater temperature, salinity, disinfection approach, stocking density etc. The questionnaires were distributed to: (a) all approved depuration establishments in the UK; and (b) industry, scientific and regulatory contacts in a variety of shellfish producing countries. The questionnaires were dispatched in 2015, and are included in Appendix 2.

3 The Depuration Process

3.1 Overview of depuration

The depuration process involves placing shellfish into tanks (custom made, or off the shelf designs), which are filled with clean seawater. During the process, shellfish should be able to filter feed normally and open and shut their valves without encumbrance, and by doing so the shellfish purge the contents of their digestive tract, including contaminants that may be associated with digested food and faecal matter. The shellfish faeces settle to the bottom of the tank and are removed following the depuration process. This natural purging process is considered to reduce the amount of most pathogenic bacteria that are present in shellfish to 'acceptable' levels.

Depuration was first investigated in the late 1800s as a method to reduce the levels of pathogenic bacteria in shellfish, in response to shellfish-associated outbreaks of cholera and typhoid fever in both the UK and USA (reviewed in Richards (1988); Richards (1991)). Some of the early systems were shown to be highly effective in reducing bacteria; for mussels and oysters, depuration reduced lactose-fermenting bacteria by 3 logs in several days (Dodgson, 1936; Richards, 1988). Since this time, depuration has been used as a post-harvest control for pathogenic bacteria in many parts of the world (including a variety of European countries, USA, Japan, China and Australia) and for a variety of bivalve species (including different species of oysters, mussels and clams). When depuration is applied using appropriate parameters and process controls, most bacteria are efficiently eliminated in relatively short timeframes, for example:

- *Salmonella enterica* serovar Typhimurium was eliminated from Pacific oysters in 12 h using a UV and chlorine based depuration system (de Abreu Corrêa et al., 2007).
- *Escherichia coli* in mussels (*Perna viridis)* was reduced to 0.2% of the starting contamination level following 15-20 h depuration (Ho and Tam, 2000).
- The bacteria *E. coli* and *Enterococcus faecalis* showed higher depuration rates in oysters and clams compared to HAV and poliovirus (Love et al, 2010).

While depuration has been found to be effective for most pathogenic bacteria, not all bacteria are depurated efficiently, with the notable exception of bacteria from the genus *Vibrio*. Richards notes in his review entitled *'Shellfish Depuration'* that "shellfish moderately contaminated with most bacterial indicators and pathogens can be adequately depurated within 72 hours". However, he further points out that bacteria of the genus *Vibrio* are indigenous in the marine environment and persist following standard depuration conditions (Richards, 1991). *Salmonella enterica* serovar Newport has also been found to persist in oysters following depuration (Morrison et al, 2012).

A variety of factors impact on the efficacy of shellfish depuration and the process must be controlled to ensure that shellfish are healthy and resume filter feeding when placed in the depuration tanks and that they are not re-contaminated during the process (Anon, 2009). The seawater used can either be from the natural environment or artificial seawater. Most depuration set-ups are based on the use of either 'flow-through' or 're-circulating' seawater, the former uses seawater that flows through the tank once and is constantly replenished with new water (hence these systems tend to be close to shore and have ready access to clean seawater), whereas re-circulating systems (common in the UK) use seawater that is re-circulated through the tank. Some bacteria that are present in the shellfish faeces (which settle to the bottom of the tank) are released into the overlying seawater; the extent of the release depends on contact time and temperature (Rowse and Fleet, 1982). The release of bacteria from the faeces can cause recontamination of shellfish, particularly those animals that are lower in the tank. Thus, depuration also involves disinfection of the seawater to prevent the build-up of bacteria and recontamination of shellfish.

A variety of different approaches for the disinfection of seawater have been used, including the use of chlorine, iodophores, ozone and ultraviolet (Richards, 1988; Richards, 1991). With respect to the use of chlorine, iodophores and ozone, shellfish are very sensitive to these compounds, which are reported to impact on shellfish pumping. Early experiments were undertaken to investigate the impact of using chlorine to disinfect the water, with results suggesting that chlorine interferes with the normal functioning of mussels and impaired purification (Dodgson, 1936). Chlorine has also been suggested to cause organoleptic changes in shellfish meat and could give rise to chlorinated by-products that are possible carcinogenic agents (Lee et al, 2008). Depuration operators can overcome these issues by de-chlorinating the seawater before adding it back into the tanks and it is more commonly used in larger flow-through depuration facilities than in closed systems. With respect to iodophors, Richards (1988) notes that systems using 0.1 to 0.4 mg/L result in bacterial reductions without impacting on shellfish activity. Ultraviolet (UV) and ozone disinfection have increased in popularity, but they have relatively high set up and running costs. Ozone can also form by-products in shellfish (e.g. bromates) and, similar to chlorine, requires operators to ensure that residual levels do not directly contact the shellfish, as this can reduce activity and depuration effectiveness (Lees et al., 2010a). UV disinfection is highly effective for bacterial reduction and has the advantage of not leaving residual concentrations and so does not interfere with the physiological processes of shellfish. Thus, UV is widely used for disinfection purposes in the USA, Australia and the UK. Key factors to ensure the efficacy of UV disinfection are the need for low turbidity, appropriate flow rate and that UV lamps are operating effectively. Thus, while all the disinfection methods can result in an effective depuration process (for bacterial contaminants), it is necessary to ensure that each technique has the appropriate controls in place.

To ensure that shellfish filter feed normally during the depuration process, a variety of parameters relating to water quality need to be considered and controlled, including dissolved oxygen levels, tank loading, water flow rate, salinity, temperature, turbidity and pH. The parameters that are used are specific to the species of shellfish to be depurated; in the UK, specific guidance and criteria have been developed.

3.2 Depuration processes in the UK

3.2.1 Regulatory requirements and guidelines for depuration in the UK

Regulation (EC) No 854/2004[1] contains the production area classification criteria and the post-harvest treatment that is required for bivalve shellfish produced in the European Union (Table 1.1). Shellfish from Class B areas must be depurated, relayed or cooked by an approved method.

[1]Criteria for classification are given in Regulation (EC) 854/2004 and Regulation (EC) 2073/2005. The criteria for Class B areas were amended by Regulation (EC) 1021/2008. The criteria for Class A areas were amended by **Regulation (EU) 2015/2285, which will be implemented in the UK on the 1st of January 2017.**

Table 1.1. Criteria for the classification of bivalve shellfish production areas.

Class	Microbiological standard	Post-harvest treatment required
A	Samples of live bivalve molluscs from these areas must not exceed, in 80% of samples collected during the review period, 230 *E. coli* per 100g of flesh and intra valvular liquid. The remaining 20% of samples must not exceed 700 *E. coli* per 100g of flesh and intra valvular liquid.	None
B	Live bivalve molluscs from these areas must not exceed, in 90% of samples, 4,600 MPN *E. coli* per 100g of flesh and intra valvular fluid. In the remaining 10% of samples, live bivalve molluscs must not exceed 46,000 MPN *E. coli* per 100g of flesh and intra valvular fluid	Depuration (purification), relaying or cooking by an approved method
C	Live bivalve molluscs from these areas must not exceed the limits of a five tube, three dilution MPN test of 46, 000 *E. coli* per 100g of flesh and intra valvular fluid	Relaying or cooking by an approved method

If shellfish harvested from Class B areas are subjected to relaying, the placement of shellfish into non-polluted seawater at the relaying site is required under the EU legislation to be for a minimum period of two months, which is considered to be enough time for elimination and inactivation of all microbial pathogens, including viruses. Regulation (EC) 853/2004 also contains other requirements for relaying. There are currently six classified relaying areas in England and Wales and none in Scotland or Northern Ireland, thus while the regulation permits relaying as a control option, it is a relatively uncommon practice in the UK and oysters harvested from Class B areas in the UK are nearly always depurated. Cooking is not generally used as an end product control option for oysters, as the consumer preference is for a raw product. Anecdotal evidence suggests that oysters harvested from Class A areas are also commonly depurated in the UK.

Regulation (EC) 853/2004 contains depuration requirements that must be adhered to, e.g.:

- Structural requirements for depuration centres i.e. tank location and structure;
- Shellfish must be free of mud and debris prior to depuration process;
- The shellfish must be able to filter feed and shell opening should not be impeded;
- The quantity of shellfish must not exceed the tank capacity;
- The depuration period is not specified in legislation but must enable compliance with the microbiological criteria contained in Regulation (EC) 2073/2005; and
- Shellfish within a depuration tank must be the same species, and crustaceans, fish or other marine species should not be kept in the depuration tank.

In the UK a variety of systems may be used for depuration, including Seafish 'standard' design systems, for which operating manuals can be consulted (http://www.seafish.org/), custom-built 'non-standard' tanks, and commercially available systems that are bought 'ready to use'. Under Regulation (EC) 853/2004 approval for purification centres must be given by the Local Authority. A series of specific requirements for purification centres has been developed in the UK by CEFAS and the FSA (Anon, 2009; Lee, 2010), these include requirements for: dissolved oxygen, tank loading, water flow rate, salinity, temperature, UV treatment, turbidity, purification period, and drain down procedure (to avoid re-suspension of sediment/faeces). A brief summary of the key water quality requirements is given below (and further summarised in Table 1.2), for full details however, the guidelines referenced above should be consulted.

Table 1.2. Summary of recommended depuration water quality criteria in the UK (summarised from Anon (2009); Lee (2010))

	Pacific oysters	Native oysters
Minimum temperature	8°C	5°C
Minimum salinity	20.5 ppt	25 ppt
Loading arrangement	Double layer	Single layer
Loading density	530 animals/m^2	
Minimum depuration period	42 hours	
Minimum UV dose	10mJ/cm^2	
UV lamps	2 x 25 watt lamps	
Dissolved oxygen	50% (5 mg/L)	

Dissolved oxygen must be adequate for shellfish to be able to function and undertake respiration normally. In the UK, dissolved oxygen levels are generally recommended to be > 5 mg/L (50%).

The way in which a tank is loaded with shellfish is important because water needs to be able to flow throughout the tank in a uniform manner ('dead spots' should be avoided), with minimum disturbance to the shellfish (i.e. from turbulence). Additionally, when shellfish filter feed they open their valves, this can be inhibited if too many shellfish are stacked on top of each other. It is recommended in the UK that the loading density of oysters in tanks not exceed 530 animals/m^2. For Pacific oysters the recommended loading arrangement is a double layer, whereas for Native oysters a single overlapping layer is recommended. The flow rate of systems varies depending on the type of system used, the type of shellfish depurated, the loading density and other factors. Seafish provide minimum recommended flow rates for standard depuration systems in the UK, all exceed 15L/minute. The specific recommended flow rate for a particular system type is specified in the approval document for each purification plant.

With respect to temperature, the metabolic rate of the shellfish is affected by the temperature of the seawater in which they are held. Generally, as temperatures decrease shellfish become less active, given this, particular temperature ranges are recommended for each shellfish species in the UK. Similarly, minimum allowable salinities are also provided. The oxygen carrying capacity of the seawater can be impacted by temperature and salinity, thus it is also important for these two parameters to be controlled to ensure shellfish can respire normally. For Pacific oysters and Native oysters respectively, minimum depuration temperatures of 8°C and 5°C are recommended in the UK, and minimum salinities of 20.5 ppt and 25 ppt are recommended.

In the UK, seawater is disinfected using UV treatment. There are several recommendations regarding the use of UV in the UK:

- The minimum applied UV dose is required to be not less than 10 mJ/cm^2 (it is recommended that higher doses or longer residence times be considered to assist in viral inactivation);
- UV lamps should be free of slime and other substances;
- The system should have 2 x 25 watt or greater UV lamps; and
- Lamps should be changed after 2500 hours of use.

In order to ensure that contaminants do not build up in the seawater, it is necessary to ensure that the seawater is of adequate transparency so that the UV can penetrate the water and inactivate microbes that may be present. If turbidity is too high, UV irradiation may be absorbed by suspended organic particles, thereby reducing the efficacy of disinfection. When turbidity is an issue, settlement or filtration of seawater may be required.

Lastly, historically a minimum depuration period of 42 hours has been recommended in the UK. The CEFAS guidelines note that "a properly functioning system should be able to reduce *E. coli* levels from ≤ 4,600 *E. coli*/100 g, to less than 80 *E. coli*/100 g in 42 hours". However, it is noted that the Hygiene Regulations do not specify a minimum period, thus from November 2016 food businesses in the UK will be able to apply alternative purification times provided they can demonstrate that the period used is appropriate.

3.2.2 Snapshot of actual industry practice in the UK

Questionnaires on oyster depuration practices and operational parameters were distributed electronically and in hard copy, where appropriate, to depuration operators identified by the FSA (information from Local Authorities). A total of 46 operators were identified across the UK (32 in England, nine in Scotland, two in Wales and three in Northern Ireland). One questionnaire 'bounced' with no alternative hard copy address available, while two respondents replied that they no longer depurated oysters. A total of 11 completed questionnaires were received out of the effective distribution of 43 forms (four in England, five in Scotland, one in Wales and one in Northern Ireland), an overall response rate of 25.6%.

The majority of systems (73%) were commissioned since 2000, with self-construction the preferred means of provision for 36% of operators. The purchased systems were predominantly 'Martin Laity' (71%), with 'Tropical Marine' and 'Depur' making up the balance (14% each). In terms of species, 36% processed only Pacific oysters, while 64% depurated both Pacific oysters and Native oysters.

Although there were a variety of systems, as detailed above, every operator used oysters loose on trays for depuration with the favoured densities ranging from 10 - 20 kg/tray for self-constructed facilities to 10 kg/tray for Depur and 15 kg/tray for the Martin Laity systems, with all of the systems falling in the range of 10 - 20 kg/tray. The manufactured systems were all limited in scale with maximum loads of 60 - 90 kg/cycle. The self-constructed systems generally exceeded these volumes, ranging from 200 kg/cycle to 1500 kg/cycle.

All respondents met or exceeded the recommended minimum depuration period of 42 hours. Elapsed time for a complete cycle was generally in the range of 42 - 48 hours (82% in summer, 73% in winter), including all manufactured systems. Self-constructed systems exhibited longer hours, typically 72 hours in summer and 96 hours in winter with one extreme example claiming a range of 42 - 120 hours in both seasons.

The majority of operators (64%) used local water sources for their supply, while 27% used artificial water and 9% relied on tanker deliveries. Filters were not installed in any of the systems, while water reuse was generally in the range of 1 - 3 times (45%), although 27% did not reuse water at all.

With respect to water disinfection, all respondents used UV disinfection with lamps exceeding 25 watts (the recommended strength for UV lamps in the UK). There were no reports of testing for turbidity, apart from a visual assessment, with several respondents noting that they followed a settlement process if turbidity was observed. Only 18% of respondents tested for microbiological contamination following the depuration process. Regarding the use of ozone for water disinfection, 91% of respondents reported never using ozone, with the balance aiming for a concentration in the range of 320 - 340 redox.

Salinity of process water ranged from 20.5 ppt (9%) through 27 - 30 ppt (27%) and 31 - 35 ppt (55%) to 40 ppt (9%). In all cases, operational temperatures were reported as ranges, with summer values ranging from minima of 8°C to 16°C and a maxima of 14°C to 22°C; winter values were minima of 2°C

to 14°C and maxima of 10°C to 18°C. Notably, some operators recorded temperatures below the UK recommended minimum values (8°C and 5°C for Pacific and Native oysters respectively) in the winter months.

The majority of respondents failed to test for, or report on, levels of dissolved oxygen in the systems (55%) and those who noted levels indicated an expectation of no change between start and end of each cycle. All respondents who reported on dissolved oxygen met, or exceeded, the recommended level of 50% (5 mg/L).

The only problems that were noted by respondents with respect to depuration operations, were difficulties in keeping the system cool in summer (with resulting spawning) (27% of respondents) and claims that depuration shortened the shelf life of the product (9%).

Depuration was generally not expected to remove NoV from oysters (55%), although 27% were willing to consider it might be possible; only 18% considered that the process offered significant potential for NoV removal. With respect to the one operator who uses ozone for disinfection, it was noted that viral testing results using PCR did not show a decrease in NoV level following the depuration process, however the company did note a decrease in customer complaints regarding illness.

3.3 Depuration processes used in other countries

Questionnaires were designed to elicit information about depuration parameters and characteristics used in a number of oyster producing countries around the world, with recipients including industry operators, regulators and research scientists. The results are therefore more 'impressionistic' than a tightly focused operators survey, however this was considered to be sufficient to highlight any major differences between UK and international practices. Sixteen responses were received, from nine countries, including: China, Netherlands, United States of America, Portugal, Norway, Ireland, New Zealand, Australia and Spain.

The first question aimed to establish the prevalence of depuration in each country. Spain, Netherlands and Portugal noted heavy reliance on depuration as a process control for oysters, with estimates of 75 - 100% of production being purified by depuration prior to placing oysters on the market. Australia (NSW), Ireland, USA and China noted that around 25 - 50% of production was subjected to depuration, Norway noted that a very small proportion of oysters were depurated (too small to quantify), and oysters from New Zealand were not subjected to depuration at all.

In terms of oyster species, seven of the eight countries in which depuration occurs noted that Pacific oysters were depurated, with the USA as the only apparent exception, with a focus on the Eastern oyster (*Crassostrea virginica*). In Portugal, a related species, *Crassostrea angulata* is also depurated in addition to Pacific oysters. Australia also depurates *Saccostrea glomerata*, the 'Sydney Rock Oyster', while local variations of 'native' oysters (*Ostrea edulis*) are also depurated in Spain and Australia.

The use of both manufactured and self-constructed depuration systems appears common in Spain, Netherlands and Ireland, while in Portugal, all systems are reported to be purchased manufactured facilities. A preference for self-constructed systems is reported for Australia (NSW), USA and China. Although the capacity of systems was generally reported as 'variable', typical capacities were identified in a number of countries, ranging from 240 kg in Portugal, to 500 kg in Netherlands and USA, to 800 kg in Australia. The implication is that the scale of depuration facilities overseas tends to be larger than in the UK, possibly due to the greater reliance on self-constructed systems (in the UK self-constructs also tend to have greater capacities than purchased systems).

Depuration cycle times varied between countries, from 24 hours in Portugal (*C. gigas* and *C. angulata*), 24 - 36 hours in Ireland (*C. gigas*), 36 hours in Australia (*C. gigas*, *S. glomerata*) and China (*C . gigas*), 36 - 48 hours in Netherlands (*C. gigas*), 44 hours in the USA (*C. virginica*), and 44 - 48 hours in Spain. These cycle times are generally shorter than UK times, with the typical time for the favoured manufactured systems being 42 - 48 hours and self-constructs reporting longer times of 72 (summer) and 96 hours (winter).

Operational temperatures varied, but within a relatively narrow band and without any species differentiation in each country. Reported temperatures were as follows: >10°C in USA, 13°C in Portugal, 13 - 15°C in Spain, 15 - 25°C in China, and 18 - 25°C in Australia (NSW). While the range of temperatures reported for the UK (from 2°C to 22°C from winter through summer) overlaps with the temperatures reported in other countries, it is notable that the winter temperatures appear to be substantively lower in the UK than those reported by the overseas respondents. This may contribute to the relatively longer depuration periods used in the UK. Salinities also varied between countries, but not between species nationally, from 15 – 31 ppt in the USA, >18 ppt in Australia, 30 ppt in the Netherlands and China, 32 – 35 ppt in Spain and 35 ppt in Portugal. These are broadly similar to those reported in the UK.

With respect to seawater disinfection approaches, similar to the UK, USA and Australia use UV disinfection routinely. The use of ozone was not widespread, with only one country (Portugal) reporting that it is consistently used in depuration facilities, and Spain and China reporting occasional use of ozone. Ozone is not used in Australia and the USA. 'Unknown' responses were recorded from Ireland and the Netherlands.

NoV illness outbreaks from oysters, which were depurated before being placed on the market, were reported by questionnaire respondents from Spain, Portugal, Australia and China, although there were no indications of the scale of the outbreaks. Several of these outbreaks have been reported in the scientific literature and are discussed in the subsequent section.

3.4 Norovirus illnesses related to depurated oysters

A recent review involved a systematic approach to identify shellfish borne viral outbreaks globally (Bellou et al., 2013). The authors interrogated a variety of different literature search engines/databases (Scopus, Pubmed etc), as well as the global electronic reporting system ProMED. The literature search yielded 61 research articles that met the inclusion criteria, and these reported on around 360 outbreaks of shellfish borne viral illness between 1980 and July 2012. The majority of outbreaks were identified in East Asia, followed by Europe. NoV was the most common virus implicated (300 outbreaks in the scientific literature), and oysters were the most frequent type of shellfish implicated (215 outbreaks reported in literature). While it is clear that there are a significant number of oyster-borne NoV outbreaks that occur globally, the review does not comment on what proportion of outbreaks resulted from oysters that had been depurated.

A major objective of this review is to evaluate the efficacy of depuration in removing NoV from oysters. Studies have been undertaken to investigate the reduction of NoV in oysters during the depuration process (see Section 5). However, one limitation of reduction studies is that they use PCR based methods for viral detection. PCR methods detect a small fragment of the viral RNA and do not provide information as to whether the virus is intact or capable of inducing infection in humans. Therefore, to provide some information on the potential infectivity of NoV in oysters after the depuration process, a literature search was undertaken to identify outbreaks that were attributed to oysters that had been depurated.

The literature search (process and keywords noted in Section 2.1) identified 1660 citations using Google Scholar and 119 citations using PubMed (but omitting the word 'depuration' from the search in PubMed to broaden the number of results). Only 15 articles met the inclusion criteria (noted in Section 2.1) for outbreaks in which oysters had been depurated. The outbreaks identified spanned the period 1979 to 2012. A further 30 articles were identified that described outbreaks attributed to oyster consumption in which the oysters either had not been depurated, or it was not stated if depuration occurred, and were thus excluded.

A summary of the oyster-borne NoV outbreaks caused by oysters that were depurated is shown in Table 1.3. Epidemiological data were presented for 12 of the 15 outbreaks. Laboratory investigations were conducted on clinical specimens (faeces) for 14 of the 15 outbreaks, and on oysters for 10 of the 15 outbreaks. In the majority of outbreaks (57%), NoV was detected in both human faecal samples and oysters.

The reported outbreaks occurred in four countries: UK, Ireland, Australia and France. In comparison, Bellou et al. (2013) notes that the reported shellfish-borne viral outbreaks (all shellfish types and viruses) occurred in 17 different countries. The smaller geographical distribution found in this review likely relates to the literature search being narrowed to only outbreaks relating to oysters that had been depurated and perhaps reflects the relatively higher implementation of depuration in the UK, France, Ireland and Australia, as compared to other countries, particularly those in East Asia.

The articles were lacking details regarding the depuration conditions used; two articles noted the seawater temperature ('ambient' and 12 - 13°C), salinity was not mentioned in any article, three articles noted the use of UV disinfection, and only one article mentioned flow rate. However, information presented in Section 3.3 on depuration conditions in different countries, suggests that the water quality parameters used to depurate oysters in the four countries implicated in these outbreaks (UK, France, Ireland and Australia) differ. For example, the temperature range reported to be used in the UK was between 2 and 22°C. For the 2012 outbreak in Australia, seawater temperature was reported to be 18°C (Anthony Zammit, Personal Communication, 2016), and the seawater temperature in the 2006 and 2008 French outbreaks was reported to be between 4 and 6°C, and 8 and 10°C respectively (Jean-Claude Le Saux, Personal Communication, 2016). Salinities used in depurating the implicated oysters were also likely to differ, with salinity in Australia reported to be quite low at >18 ppt (Section 3.3), whereas 55% of respondents to the survey undertaken as part of this review noted that salinity was between 31 and 35 ppt in the UK (Section 3.2). UV disinfection is ubiquitously used in the UK and Australia (57% of the outbreaks came from these countries). For the 2006 French outbreak, UV was also used, but in the 2008 outbreak a recirculating depuration system was used with aeration only (Jean-Claude Le Saux, Personal Communication, 2016). The variety of depuration conditions used in the purification process for oysters responsible for the 15 outbreaks displayed in Table 1.3 may imply that altering water quality parameters such as salinity, temperature, flow rate, and stacking density will not be sufficient to reduce NoV to levels that are below an infectious dose. Further information on viral reduction studies in which the alteration of water quality parameters has been investigated are presented in Section 5.

For six of the outbreaks the concentration of NoV following depuration was reported; concentrations varied between 1.7×10^2 and 8×10^3 NoV genome copies/g. Concentrations of NoV in oysters were not reported pre 2002, largely due to the lack of methods that enable accurate quantification to be carried out. Recent estimates of the infectious dose of NoV suggest that it is very low (Atmar et al., 2014; Teunis et al., 2008) (Section 4). Thus, it is not surprising that oysters containing between 1.7×10^2 and 8×10^3 viral genomes were implicated in gastroenteritis outbreaks, and this demonstrates that a proportion of the viruses present in the oysters were capable of inducing infection and illness despite the depuration process being applied. As discussed above, in at least three of the reported

outbreaks the depuration process involved UV treatment (Gill et al., 1983; Grohmann et al., 1981; Heller et al., 1986), and in one of the outbreaks an extended depuration period was used, consisting of 15 days (Heller et al., 1986). These measures were clearly ineffective in reducing NoV to 'safe' levels.

While undertaking the literature search on outbreaks of NoV related to depurated oysters, two additional articles on outbreaks of Hepatitis A related to the consumption of depurated oysters were identified (Table 1.3) (Conaty et al., 2000; Guillois-Becel et al., 2009). Epidemiological data were provided for both outbreaks and HAV was confirmed in the sera of patients from one outbreak and in the oysters of the second outbreak. Both outbreaks involved the depuration of the oysters for relatively short periods, 36 and 48 hours.

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Table 1.3. Published outbreaks of NoV illness related to the consumption of depurated oysters

Number of cases	Year	Oyster species	Country of origin	Epidemiological linkage	Virus detected in faeces by PCR	Virus detected in oysters by PCR	Virus copies/g in oysters (post depuration)[4]	Depuration time	Reference
NoV outbreaks									
18	2012	Pacific oysters	Ireland	NS	Y (GI and GII)	Y (GI and GII)	2.38 x 10^3	NS	Rajko-Nenow et al. (2014)
8	2012	NS	Australia	Y	Y (GII)	Y (GII)	NS	36 hours	Fitzgerald et al. (2014)
70	2010	Pacific oysters	Ireland	NS	Y (GII)	Y (GI and GII)	2.35 x 10^3	NS	Dore et al. (2010); Rajko-Nenow et al. (2014)
≥ 240	2009	NS	UK[1]	Y	Y (GI and GII)	Y (GI and GII)	NS	NS	Smith et al. (2012)
23	2008	Pacific oysters	France	Y	Y (GII)	Y (GII)	2.4 x 10^3	1-23 days[5]	Le Guyader et al. (2010)
>93	2007	Pacific oysters	UK	NS	Y (GII)	Y (GII)	8 x 10^3	NS	Lowther et al. (2010)
19	2007	NS	Australia	Y	Y	N	NA	36 hours	Huppatz et al. (2008)
205	2006	Pacific oysters	France	Y	Y (GI and GII)[2]	Y (GI and GII)[2]	2.4 x10^3	1-12 days[5]	Le Guyader et al. (2008)
15	2004	NS	UK	Y	Y (GI and GII)	NT	NA	42 hours	Gallimore et al. (2005)
329	2002	Pacific oysters	France	Y	Y (GI and GII)	Y (GI and GII)	1.7 x 10^2	48 hours	Le Guyader et al. (2006b)
9	1997	Pacific oysters	UK	Y	Y[3]	NT	NA	NS	Ang (1998)
97	1996	NS	Australia	Y	Y (GII)	N	NA	NS	Stafford et al. (1997)
16	1985	Pacific oysters	UK	Y	N	NT	NA	15 days	Heller et al. (1986)
181	1983	Pacific oysters	UK[1]	Y	Y[3]	NT	NA	72 hours	Gill et al. (1983)
52	1979	*Saccostrea glomerata*	Australia	Y	Y	NT	NA	48 hours	Grohmann et al. (1981)
HAV outbreaks									
111	2007	Pacific oysters	France	Y	Y (sera)	N	NA	48 hours	Guillois-Becel et al. (2009)
467	1997	*Saccostrea glomerata*	Australia	Y	NT	Y	NS	36 hours	Conaty et al. (2000)

Y = yes; N = No; NS = Not Stated; NA = Not Applicable; NT = Not tested

[1]Outbreak in UK, location of supplier not noted.

[2]Other enteric viruses were also detected in the oysters and faeces, including Aichi virus, Astrovirus, Enterovirus and Rotavirus

[3]Small round structured viruses (SRSV) were visualised in faecal samples.

[4]Where multiple values reported, the maximum value is recorded in the table. Where possible, the values reported are from samples collected from either the restaurant or the consumer (not harvesting area).

[5]Depuration time for oysters consumed and implicated in the outbreak were not stated in the publication. However, information obtained from the authors provided a range of days that oysters implicated in the outbreak were depurated for

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4 Norovirus – Oyster Interactions

When considering the efficiency of depuration and relaying in reducing NoV in oysters, it is necessary to understand how NoV is ingested, retained and excreted by oysters, because this may provide some explanation for the observed illnesses relating to oysters that have been depurated (Section 3), and for the slow elimination rates reported for NoV in oysters (Section 5). Thus, the following section presents basic biological information on NoV, uptake of NoV by oysters, and the state-of-the-art with respect to specific ligands that are present in oysters that act to selectively concentrate and retain NoV in the oyster digestive tract and other tissues.

4.1 Norovirus

NoVs are a group of highly diverse viruses that belong to the *Caliciviridae* family. They have a single stranded RNA genome, which is around 7500 bp long, and they are non-enveloped and icosahedral viruses (Le Guyader et al., 2012). NoV causes gastroenteritis, the symptoms often include vomiting, abdominal cramps, fever, watery diarrhoea, headaches, chills and myalgia, and illness normally lasts two to three days (Glass et al., 2009). NoVs infect humans by binding to histo-blood group antigens (HBGAs), these are highly conserved glycans (carbohydrates) which are present on a wide variety of gastrointestinal and epithelial cell types and are used as receptors by many viruses and bacteria (Le Pendu et al, 2014).

There are currently seven genogroups, of which three infect humans (GI, GII and GIV) (Zheng et al., 2006). NoV is the main cause of non-bacterial gastroenteritis outbreaks worldwide, with GII.4 strains responsible for the majority of outbreaks (Glass et al., 2009; Koopmans, 2008; Siebenga et al., 2010). In a recent review by Le Guyader et al. (2012), information on NoV genotypes detected in stools and shellfish implicated in illness outbreaks was collated; this demonstrated that the frequency of detection of genogroups in shellfish related outbreaks is different, with GI strains more frequently detected in shellfish outbreaks compared to other NoV outbreaks. Similarly, Yu et al. (2014) found that there are more GI sequences reported (NCBI GenBank and the NoroNet outbreak database) from oyster outbreaks (34%), than from non-oyster outbreaks for which 90% are GII strains.

The infectious dose of NoV is considered to be very low, a human trial involving a GI.1 strain determined that the average probability of infection for a single viral genome was 0.5 and the median infectious dose (ID_{50}) was between 18 and 1015 genome copies (Teunis et al., 2008). A separate human trial was recently conducted in USA also involving a GI.1 strain, which reports an ID_{50} of approximately 1320 genome copies for secretor positive persons who were blood type O or A (Atmar et al., 2014). Thebault et al. (2013) statistically analysed data from five published outbreaks resulting from NoV in oysters in France. Median ID_{50} estimates ranged between 1.6 and 7.51 genome copies per oyster consumed and the probability of infection of a single NoV genome copy was close to 0.5 for both GI and GII NoV, suggesting that there is no difference in the infectivity between GI and GII NoVs (Thebault et al., 2013).

4.2 Contamination of oyster production areas and oyster feeding

There are two major routes by which food contamination occurs, through infected food handlers and during the primary production process i.e. through contact of the food with sewage contaminated water. In the case of oysters, contamination primarily occurs in the production area when the seawater becomes contaminated with sewage. Contamination of oyster production areas with sewage can occur in a number of ways, including:

- Through the release of partially-treated or non-treated sewage from wastewater treatment plants or broken sewerage pipes and pump stations (Doyle et al., 2004; Guillois-Becel et al., 2009; Maalouf et al., 2010a);

- Via leachate from septic tanks (on-site sewage disposal units) (Stafford et al., 1997);
- Following high rainfall or flood events in which contaminated run off water pollutes the production area (Conaty et al., 2000; Doyle et al., 2004; Grodzki et al., 2012; Le Guyader et al., 2008; Murphy and Grohmann, 1980);
- From harvesters and/or other people defecating directly into production areas (Berg et al., 2000; Kohn et al., 1995; McDonnell et al., 1997; McIntyre et al., 2012); or
- Through release of sewage by recreational or commercial vessels (Simmons et al., 2001).

Oysters are filter feeding bivalve molluscs. They grow in both intertidal and subtidal areas, however they prefer estuarine areas close to the shore. The gills of the oyster are involved in the capture, selection and transport of food particles, as well as respiration. The cilia on the gills create water currents, which draw seawater across the gills. Mucus on the gills binds particles that are present in the seawater, which are then carried forward to the labial palps and mouth. Unwanted particles are rejected in the pseudofaeces prior to ingestion (Ward et al., 1997, 1998). Viruses are found in both pseudofaeces and faeces, however a higher proportion of non-culturable HAV and PV were detected in faeces compared to pseudofaeces, indicating that viruses are inactivated as they transit the oyster digestive system (McLeod et al., 2009a). Factors such as nutritional value, size and charge of food particles are thought to influence the selection of food by bivalves (Bedford et al., 1978; Shumway et al., 1985; Ward et al., 1997). Additionally, recent research has demonstrated that there is an interaction between carbohydrates on the algal cell surface and lectins within the mucus that covers the feeding organs of mussels (*Mytilus edulis*) and oysters (*Crassostrea virginica*) (Espinosa et al., 2010a; Espinosa et al., 2009, 2010b), and this is suggested to be a common mechanism for particle selection across bivalve taxa.

Pacific oysters can efficiently capture food particles in the 4 to 10 µm size range (Bell, 2005). However, oysters also retain smaller particles, such as NoV (which is around 23 nm in size). A study undertaken in the late 1970s demonstrated that the bioaccumulation efficiency of poliovirus by clams increased when the virus was added to the seawater in conjunction with clay kaolinite or faeces (Metcalf et al., 1979). More recent research demonstrates the presence of NoV in plankton samples (Gentry et al., 2009). Thus, the adhesion of viruses to solids, including plankton, may enhance bioaccumulation in shellfish. It is also likely that viruses bind to the mucus that flows through the gills and labial palps of the oyster. Early research suggested that binding of viruses to the mucous sheath was ionic in nature (Di Girolamo et al., 1977), however given the recent findings regarding the binding of algae to lectins within oyster mucus it seems feasible that carbohydrates on the surface of the NoV capsid may also bind to lectins within the oyster mucus (though this hypothesis remains to be confirmed). Further research has also shown the direct interaction of NoV with oyster ligands present on/in the gills and other digestive structures, as discussed in the next section.

4.3 Interaction of Norovirus with oyster ligands

The recognition that NoV persists for longer periods than bacteria when oysters are subjected to depuration or relaying (Schwab et al., 1998; Ueki et al., 2007) led to suggestions that NoV may be binding specifically to oyster tissues, thus increasing the amount of time that the virus remains in the oyster and prompting investigations to identify ligands that NoV may be adhering to within the oyster.

Firstly, a GI.1 strain was shown to bind to the midgut and digestive diverticula of Pacific oysters, but not to the other tissue types (Le Guyader et al., 2006a). In contrast GII NoV was shown to bind to a variety of oyster tissue types, including the digestive diverticula, midgut (intestine), gills, mantle, and labial palps (McLeod et al., 2009b; Seamer, 2007; Wang et al., 2008). Collectively these results suggested strain specific variations in binding patterns.

Secondly, Le Guyader et al. (2006a) determined that binding of GI.1 NoV to Pacific oyster tissues was

inhibited by human saliva from type A and O secretors, and that a mutation in the glycan binding site of NoV virus-like-particles (VLPs) prevented them from binding to oyster tissues. This contributed to the conclusion that GI.1 NoV was binding to oyster tissues via an A-like carbohydrate structure, similar to the HBGAs used for NoV attachment to human epithelial cells. Similar results were obtained by a different research group, who showed that binding of GI.1 NoV to *Crassostrea virginica* (the Eastern oyster) was inhibited by anti-blood group A antibodies (Tian et al., 2006), also supporting the hypothesis that binding occurs through an A-like antigen.

The binding of GII NoV to oyster digestive tissues was also shown to occur through an A-like antigen, but binding of GII strains to gills and mantle tissue is facilitated by a sialic acid residue (Maalouf et al., 2011; Maalouf et al., 2010b). This bioaccumulation study also confirmed that GII NoV strains bind to a number of tissues, but GI strains are confined to binding to the digestive tissue (Maalouf et al., 2011). Furthermore, the GII.3 strain showed transient expression in the gills and mantle before being almost exclusively localised in the digestive tract. The authors hypothesised that this observation was due to the binding of GII.3 to a sialic acid ligand in the gills and mantle, which facilitated destruction of the virus (Maalouf et al., 2011).

Further bioaccumulation studies conducted at different times of the year demonstrated that there is a seasonal impact with respect to accumulation of GI NoV in oysters; this is mirrored by the expression of the A-like HBGA ligand in oyster digestive tissue. In contrast, no seasonal effect was observed in bioaccumulation studies of GII NoVs in oysters or of the sialic acid ligand (Maalouf et al., 2010b).

Thus oysters are not just passive filters, but they use specific ligands to selectively accumulate NoV. GI and GII NoV strains are considered to bind to A-like antigens in the digestive tissue, which facilitate their accumulation in oysters. This specific binding may help to explain their prolonged retention, as observed in depuration and relaying studies to date (Section 5) and account for illness outbreaks attributed to depurated oysters (Section 3). In contrast, the binding of GII strains to a sialic acid ligand in the mantle and gills is hypothesised to facilitate their elimination from oysters. For further information, Le Guyader et al. (2012) describe these issues in more detail in a recent review on the transmission of viruses through shellfish.

4.4 Current detection method for Norovirus in oysters

A variety of methods have been published for the detection of NoV in shellfish (Atmar et al., 1995; Boom et al., 1990; Greening and Hewitt, 2008; Henshilwood et al., 1998; Lees, 2010b; Shieh et al., 2000; Shieh et al., 1999b; Sobsey et al., 1985). The main challenge is to remove inhibitors of the polymerase chain reaction (PCR), which are found in the shellfish, such as glycogen. NoV is primarily concentrated in the oyster digestive tract (McLeod et al., 2009b), which is dissected from the oyster and the entire surrounding white gonad tissue is discarded. Methods for recovering the virus from the oyster digestive tract have included approaches such as alkaline elution using glycine buffer (Traore, 1998), acid adsorption (Shieh et al., 1999a), and protease digestion (Jothikumar et al., 2005). Some methods incorporate polyethylene glycol to concentrate the virus (Lewis and Metcalf, 1988). Following the evaluation of a variety of methods, a standard method has been developed and validated under the auspices of the European Committee on Standardisation (CEN) (Lees, 2010b). Two approaches have been developed: standard method ISO/TS 15216-1:2013, which is quantitative, and ISO/TS 15216-2:2013, which is qualitative. The standard methods were published in 2012 and incorporate protease digestion for virus recovery, followed by guanidine thiocyanate and silica adsorption to purify the RNA. The method uses real-time PCR to detect a small fragment of the viral genome sequence targeting the conserved region at the 5' end of ORF2. The main drawback of the currently available methods, including the ISO standard approaches, is that they do not differentiate between infectious and non-infectious virus particles. While the methods can be used quantitatively

by incorporating nucleic acid standards, there are many sources of variation that affect results and determining small differences in virus concentrations (i.e. within a log) between samples can prove challenging.

5 Persistence of Norovirus in Oysters

5.1 NoV persistence in oysters in the natural environment following relaying

Several studies have investigated the efficiency with which NoV is eliminated from Pacific oysters in the natural environment (open seawater) over an extended period. Table 1.4 presents a summary of NoV levels recorded in oysters prior to and after relaying and depuration.

Le Guyader et al. (2008) investigated the reduction of GI and GII NoV in oysters (*C. gigas*) that were associated with 205 cases of gastroenteritis in France. The illness outbreaks occurred following a flood event in the implicated production area when flood oysters were collected over a four-week period. GI and GII NoV fell from 3 log genome copies/g to around the LoQ over the four-week period, for which seawater temperatures were between 8 and 10°C. Similarly, Dore et al. (2010) studied the combined use of relaying (17 days) and in-tank depuration (six days) to reduce GII NoV in oysters (*C. gigas*) that were implicated in around 70 cases of illness. Levels declined from 2.9 x 10^3 genome copies/g to 492 copies/g following the 17-day relaying period (seawater temperature 15 - 17°C), and fell to below the LoQ after a further six days of depuration. Greening et al. (2003) artificially contaminated Pacific oysters with GII NoV and then suspended the oysters in open clean seawater (18 - 12°C) for a period of six weeks. Initial concentrations were around 4 log PCR units/g and NoV was able to be detected through to week 4, but not after this period of time.

All studies which have evaluated the reduction of NoV in oysters in the natural environment have relied on PCR methods to detect the virus as there is no culture-based method which is amenable to the routine analysis of shellfish for NoV. Thus, there is only indirect information available about the infectivity of NoV following relaying, such as data from studies on the elimination of culturable viruses from oysters over extended periods of time.

Two trials were undertaken on the elimination of FRNA bacteriophage during four weeks of relaying in ponds, followed by in-tank depuration for 48 h. For Pacific oysters, phage was reduced to below the LoD in two weeks in one trial, but low levels remained after four weeks of relaying and depuration in the second trial (Dore et al., 1998). The persistence of HAV in oysters (*C. virginica*) maintained in depuration tanks (recirculating system with UV treatment) was tracked over an extended four-week period. Seawater temperature was 18°C and microalgae were added to the tanks as a food source. HAV was still able to be cultured three weeks after the oysters were contaminated, but not after four, five and six weeks of depuration. In contrast, HAV RNA was detected following six weeks of depuration (Kingsley and Richards, 2003).

Collectively, these studies suggest that a relay period of around four weeks is sufficient to reduce GI and GII NoV and HAV to background levels in Pacific oysters, and that viral infectivity is significantly reduced after this period of time.

Dore et al. (2010) and Le Guyader et al. (2008) both reported that NoV was still detectable in some samples following three to four weeks of purification in open seawater. In both cases oysters were allowed to be sold for consumption following the cleansing process and no further human illnesses were reported. This may imply that risk to consumers is small from low levels of NoV in oysters which have been purified for three to four weeks in clean open seawater. However, the presence of culturable phage (at low levels) following four weeks of purification (Dore et al., 1998) indicates the possibility that a small number of NoV particles may be capable of inducing infection following relaying; it is possible that such low levels of contamination either results in no illness (as it is below the infectious dose), or in such small numbers of cases that they are not identified/reported through epidemiological surveillance systems.

5.2 NoV and HAV persistence in oysters following 'in-tank' depuration

Studies that have investigated the reduction of NoV (GI and GII) in *C. gigas* in depuration tanks have demonstrated that periods of between 23 h and 14 days result in no or negligible reductions of NoV (Table 1.4). McLeod et al. (2009a) demonstrated no reduction of GII NoV over 23 h in Pacific oysters maintained in a re-circulating system at 20°C, likewise Schwab et al. (1998) did not observe any differences in GI NoV level in Eastern oysters before and after 48 h depuration at 22°C. No drop in GII NoV levels were observed over a 10 day period in a flow through system maintained at 10°C (Ueki et al., 2007), or over a 14 day period for Pacific oysters maintained at 8°C in a commercial system with UV disinfection (Neish, 2013). Reductions of around 0.5 log GI NoV were achieved over eight days in a system maintained at 8 - 10°C (Drouaz et al., 2015), likewise a 0.5 log reduction was demonstrated for GII NoV in a system maintained at 16°C (Neish, 2013). Thus, depuration periods of less than two weeks at a variety of temperatures ranging from 8 - 22°C appear to have limited impact on NoV concentrations (Table 5.1).

Several studies investigated the reduction of GI NoV in Pacific (*C. gigas*) and Eastern oysters (*C. virginica*) in depuration tanks over extended periods (five - eight weeks) (Table 1.4). Drouaz et al. (2015) maintained Pacific oysters that were contaminated with 3.8 x 10^4 copies/g of GI NoV for eight weeks in filtered re-circulating seawater at 11°C, levels reached the LoQ by week 8. The reduction of GI NoV was also monitored in Eastern oysters maintained at three temperatures, 7, 15 and 25°C, in depuration tanks with re-circulating UV treated seawater over a six-week period. NoV decreased from 6 log copies/oyster to 4 log copies/oyster over six weeks at 7 and 15°C, but was not detected beyond week 4 in oysters at 25°C, clearly showing that elevated temperatures can enhance depuration efficiency (Choi and Kingsley, 2016).

Table 1.5 presents a summary of HAV levels recorded in oysters prior to and after depuration. Regarding HAV, depuration for 23 h at 20°C in a recirculating system did not result in a significant loss/drop in HAV genomes or plaque forming units (PFU) (McLeod et al., 2009a). Similarly, Love et al. (2010) demonstrated modest reductions of HAV in *C. virginica* depurated for five days at 12 and 18°C, but a significantly higher depuration rate was identified at 25°C, with around 98.5% (<1 log) eliminated in 44 h, again demonstrating that higher temperatures can enhance depuration in Eastern oysters.

To compare the reduction rate of NoV and HAV across different studies, viral levels in oysters were determined for each study from either raw data reported in publications, or estimated from graphs using WebPlotDigitizer (http://arohatgi.info/WebPlotDigitizer/). Similar to other authors (Choi and Kingsley, 2016; Loisy et al., 2005; Love et al., 2010), it was assumed that viral loss was exponential and a linear regression model was fitted to the estimated $\log_{10}$ transformed NoV concentrations to assess the reduction in NoV and HAV over time (Excel, 2016, Microsoft®). The models were then used to predict an estimated average single log reduction time for NoV and HAV within oyster tissues for each published study (Tables 1.4 and 1.5).

Of the 16 published NoV reduction experiments, the observed loss of NoV genomes in eight experiments was too limited to allow a prediction of a log reduction time (i.e. no or very low reductions observed). The estimated days to achieve 1 log reduction of NoV (genomes) in the eight remaining experiments was between nine and 45.5 days (Table 1.4). For HAV, the estimated days to achieve 1 log reduction was between seven and 16.1 days, with two of the five experiments showing no loss of HAV (Table 1.5). A range of factors may influence the rate at which NoV and HAV are purged by oysters, including the oyster species involved, seawater temperature and salinity, the presence or absence of food for the oysters, the length of the contamination period and initial level of contamination. This is discussed further in Section 5.5.

Table 1.4. NoV levels reported for oysters following purification and the number of days of purification estimated to achieve a one log reduction.

NoV Genogroup	Oyster species	Purification period	Seawater temp	Type of purification	Seawater treatment	Feeding during depuration	Virus concentration post accumulation	Virus concentration post depuration	Days to achieve 1 log reduction	Author
Relaying experiments										
GI and GII	*C. gigas*	4 weeks	8 – 10 °C	R	NA	NA	8.2 x 10^3 copies/g	420 copies/g	8.9	Le Guyader et al. (2008)
GII	*C. gigas*	6 weeks	18 - 12 °C	R	NA	NA	1 x 10^4 PCR units/g	Not Detected	10.6	Greening et al. (2003)
GII	*C. gigas*	17 days	15 – 17 °C	R	NA	NA	2.9 x 10^3 copies/g	4.9 x 10^2 copies/g	23.1	Dore et al. (2010)
GII	*C. gigas*	17 days R + 6 days D	15 - 17 °C	R + D	NA NS	NA NS	2.9 x 10^3 copies/g	<100 copies/g	16.6	
Depuration experiments										
GII	*C. gigas*	23 hours	20°C	RC	None	No	2.7 x 10^4 PCR units/g	3.9 x 10^4 PCR units/g	LR	McLeod et al. (2009a)
GI	*C. virginica*	48 hours	20 - 24°C	FT	NA	No	792 PCR units/oyster	734 PCR units/oyster	LR	Schwab et al. (1998)
GI	*C. gigas*	8 days	8 – 10°C	NS	NS	No	1.4 x 10^4 copies/g	4.6 x 10^3 copies/g	LR	Drouaz et al. (2015)
GI	*C. gigas*	8 days	8 – 10°C	NS	NS	Yes	5.9 x 10^4 copies/g	7.6 x 10^3 copies/g	LR	
GII	*C. gigas*	10 days	10°C	FT	Filtration	No	a. 1.7 x 10^3 copies/g b. 5.2 x 10^3 copies/g	a. 1.8 x 10^3 copies/g b. 7.7 x 10^3 copies/g	LR	Ueki et al. (2007)
GII	*C. gigas*	14 days	8°C	RC	UV	No	1.7 x 10^5 copies/g	2.4 x 10^5 copies/g	LR	Neish (2013)
GII	*C. gigas*	14 days	16°C	RC	UV	No	1.7 x 10^5 copies/g	1.1 x 10^5 copies/g	45.5	
GI	*C. gigas*	8 weeks	11°C	RC	Filtration	Yes	3.8 x 10^4 copies/g	<100 copies/g	22.6	Drouaz et al. (2015)
GI	*C. virginica*	5 weeks	25°C	RC	UV	No	1-2 x 10^6 copies/oyster	Not Detected (100 copies at 4 weeks)	10.15	Choi and Kingsley (2016)
GI	*C. virginica*	6 weeks	15°C	RC	UV	No	1-2 x 10^6 copies/oyster	1 x 10^4 copies/oyster	15.85	
GI	*C. virginica*	6 weeks	7°C	RC	UV	No	1-2 x 10^6 copies/oyster	3.8 x 10^4 copies/oyster	LR	

GII = genogroup 2 NoV; GI = genogroup I NoV; R = relaying; D = depuration; RC = recirculating system; FT = flow through system; NA = not applicable; NS = not stated in publication; LR = limited reduction, observed loss of NoV genomes too low to allow a prediction of a log reduction time.

Table 1.5. HAV levels reported for oysters following depuration and the number of days of purification estimated to achieve a one log reduction.

Oyster species	Depuration period	Seawater temperature	Type of depuration	Seawater treatment	Feeding during depuration	Virus concentration post accumulation	Virus concentration post depuration	Days to achieve 1 log reduction	Author
C. gigas	23 h	20°C	RC	None	No	1.2 x 10^5 PCR units/g 8.4 x 10^4 PFU/g	1.1 x 10^5 PCR units/g 4.7 x 10^4 PFU/g	LR	McLeod et al. (2009a)
C. virginica	5 days	25°C	FT	NA	No	NS	NS	13.6	Love et al. (2010)
C. virginica	5 days	18°C	FT	NA	No	NS	NS	16.1	
C. virginica	5 days	12°C	FT	NA	No	NS	NS	LR	
C. virginica	6 weeks	18°C	RC	UV	Yes	3.4 x 10^4 PFU/ml shellfish extract	Not Detected (500 PFU at 3 weeks)	7	Kingsley and Richards (2003)

RC = recirculating system; FT = flow through system; NA = not applicable; NS = not stated in publication; LR = limited reduction, observed loss of HAV too low to allow a prediction of a log reduction time.

5.3 Persistence of surrogate viruses during depuration

As previously noted, NoV is unable to be reliably detected/quantified in shellfish tissues using cell culture methodologies. Thus while it is clear that NoV genomes can persist for prolonged periods, it is not known how long infectivity of NoV is retained within oyster tissues. To overcome this problem, studies have been conducted to evaluate the usefulness of surrogate viruses (which are generally able to be cultured) for assessing the virological safety of depurated oysters. Table 1.6 presents a summary of depuration studies conducted to date using surrogate viruses in oysters. For a surrogate virus to provide useful information on the infectivity of NoV, it is important that the characteristics of NoV and the surrogate virus are similar within oyster tissues, including the way in which they interact with ligands, the stability of the virus capsid and their persistence.

Several studies have been undertaken on the usefulness of FRNA bacteriophage (MS2) as an NoV surrogate during depuration and relaying. Love et al. (2010) followed the depuration of FRNA bacteriophage (MS2), poliovirus and HAV from *C. virginica* over five days at 25°C in a flow through system. Depuration rates, as determined using culture methods for each virus, were the greatest for phage, followed by poliovirus (PV), then HAV; days to achieve a 1 log reduction of each virus were estimated to be 2.1, 6.9 and 13.6 days respectively (Tables 1.6 and 1.5). Because FRNA bacteriophage and PV were removed at faster rates than HAV, it appears they may be poor indicators of the virological status of depurated oysters. Consistent with these results, Loisy et al. (2005) found that MS2 phage was depurated more rapidly than rotavirus VLPs from Pacific oysters, with the time to achieve a 1 log reduction estimated to be 4.1 and 6.6 days respectively (Table 1.6).

Neish et al. (2013) also undertook depuration experiments using Pacific oysters and FRNA bacteriophage. No reduction of NoV was noted at 8°C over 14 days, but phage was reduced by nearly 1 log. Similar results were achieved at the higher temperature of 16°C, with days estimated to achieve a 1 log reduction of 6.5 for phage, and 45.5 for NoV. The difference in depuration rates between phage and NoV appear vast in this study. However, phage was detected using a culture method thus only infectious virus was detected, whereas NoV was detected using PCR which detects infectious and non-infectious particles and may underestimate reductions in infectivity. Nonetheless, the results are indicative of large differences in the behaviour of phage and NoV during depuration, and are consistent with the differences in depuration rates noted between phage and HAV (for which culture methods were used for both viruses).

Ueki et al. (2007) conducted a parallel study to investigate the comparative depuration rate of NoV and feline calicivirus (FCV) – a potential NoV surrogate. Following 72 h contamination, oysters (*C. gigas*) were depurated for 10 days at 10°C in a flow through system with sand filtration. NoV levels did not decrease, whereas FCV was rapidly depurated within three days. This is consistent with the results of Provost et al. (2011), who used RT-PCR to demonstrate that FCV was undetectable one day after contamination of oysters (*C. virginica*), whereas HAV was able to be detected for >21 days. Murine norovirus (MNV) showed an intermediate persistence and was detected for up to 12 days.

McLeod et al. (2009a) compared the elimination rates of HAV, NoV and PV in Pacific oysters over 23 h of depuration in a re-circulating system. While there was no decline in HAV and NoV genome copies in oysters, there was around a 2 log decrease of PV genome copies (and a 1 log decrease in PFU). Love et al. (2010) and Provost et al. (2011) also showed that PV was removed at a faster rate than HAV in *C. virginica.*

Drouaz et al. (2015) investigated the comparative depuration of Tulane virus (TV), Mengovirus (MgV) and NoV (GI) from Pacific oysters when maintained in large scale commercial depuration tanks at 11°C for eight weeks. Oysters were fed phytoplankton throughout the trial and samples were

collected weekly. PCR methods were used for the detection of all three viruses. NoV (GI) was found to be more persistent in oysters than TV or MgV, with half-lives of 7.56 days, 4.65 days and 2.17 days respectively. The authors hypothesised that TV may behave more like a NoV GII strain (rather than GI trialled in the study), due to differences in the HBGAs that GII and GI strains bind to.

In summary, comparative elimination studies to date have shown that surrogate viruses are more rapidly depurated than NoV and HAV under a variety of depuration conditions, including temperatures of 8 - 25°C, times varying between 23 h and eight weeks, and using both recirculating and flow through systems which have UV and or filtration disinfection. As part of this review, the time required to reduce NoV and HAV by 1 log in each depuration study was estimated (Tables 1.4 and 1.5), this was also estimated for the surrogate viruses (Table 1.6). A comparison of the days to achieve 1 log reduction, shows that the number of days to reduce NoV/HAV (mean = 19 days for NoV, 12 days for HAV) is greater than that recorded for the surrogate viruses (mean = 7.5 days). Given the more rapid depuration of indicator viruses tested to date, they may not be suitable surrogates for assessing the virological safety of depurated oysters.

Table 1.6. Levels of surrogate viruses in oysters following depuration and relaying and the number of days of purification estimated to achieve a one log reduction.

Virus type	Oyster species	Depuration period	Seawater temperature	Type of depuration	Seawater treatment	Feeding	Virus concentration post accumulation	Virus concentration post depuration	Days to achieve 1 log reduction	Author
Phage (salmonella WG49 host)	*C. gigas*	7 days	9°C	NS	NS	No	a. 3.4 x 10^4 PFU/100g b. 2.8 x 10^3 PFU/100g	a. 1.4 x 10^4 PFU/100g b. 4.2 x 10^2 PFU/100g	a. 1.8 b. LR	Dore et al. (1998)
	C. gigas	7 days	18°C	NS	NS	No	a. 3.4 x 10^4 PFU/100g b. 2.8 x 10^3 PFU/100g	a. 6.6 x 10^2 PFU/100g b. <30 PFU/100g	a. 4.8 b. 1.0	
	C. gigas	4 weeks R + 48 h D	NS	R+D	NS	No	a. 1.9 x 10^4 PFU/100g b. 1.8 x 10^4 PFU/100g	a. <30 PFU/100g b. 6 x 10^2 PFU/100g	a. 5.0 b. 18.9	
	O. edulis	4 weeks R + 48 h D	NS	R+D	NS	No	a. 2 x 10^4 PFU/100g b. 2.9 x 10^3 PFU/100g	a. <20 PFU/100g b. <30 PFU/100g	a. 5.0 b. 14.1	
Phage (salmonella WG49 host)	*C. gigas*	14 days	8°C	RC	UV	No	9 x 10^3 PFU/g	1.2 x 10^3 PFU/g	14.6	Neish (2013)
	C. gigas	14 days	16°C	RC	UV	No	9 x 10^3 PFU/g	65 PFU/g	6.5	
Phage MS2 (*E. coli* host)	*C. gigas*	7 days	22°C	RC	F + UV	No	2 x 10^3 PFU/g	42 PFU/g	4.1	Loisy et al. (2005)
Phage MS2 (*E. coli* host)	*C. virginica*	5 days	25°C	FT	NA	No	NS	NS	2.1	Love et al. (2010)
Poliovirus	*C. virginica*	5 days	25°C	FT	NA	No	NS	NS	6.9	
Poliovirus	*C. gigas*	23 h	20°C	RC	None	No	1.2 x 10^5 PCR units/g 5.8 x 10^3 PFU/g	8.5 x 10^3 PCR units/g 1.7 x 10^3 PFU/g	0.6 1.7	McLeod et al. (2009a)
Rotavirus virus like particles	*C. gigas*	7 days	22°C	RC	F + UV	No	1.1 x 10^6 particles/g	1 x 10^5 particles/g	6.6	Loisy et al. (2005)
Tulane virus	*C. gigas*	8 days	8 – 10°C	NS	NS	No	3 x 10^3 copies/g	1.2 x 10^3 copies/g	LR	Drouaz et al. (2015)
Tulane virus	*C. gigas*	8 days	8 – 10°C	NS	NS	Yes	6.9 x 10^4 copies/g	1.1 x 10^4 copies/g	LR	
Tulane virus	*C. gigas*	8 weeks	11°C	RC	F	Yes	6.7 x 10^4 copies/g	<100 copies/g	11.5	
Mengovirus	*C. gigas*	8 weeks	11°C	RC	F	Yes	1.5 x 10^5 copies/g	<100 copies/g	11.4	
Feline calicivirus	*C. gigas*	10 days	10°C	FT	Filtration	No	a. 2.2 x 10^3 copies/g b. 5 x 10^3 copies/g	Not detected Not detected	<0.9	Ueki et al. (2007)

R = relaying; D = depuration; NS = not stated in publication; RC = recirculating system; FT = flow through system; F = Filtration; UV = ultra violet radiation; LR = limited reduction, observed loss did not allow a prediction of a log reduction time.

5.4 Possible reasons for variation in the persistence of different types of viruses

Comparative studies demonstrate significant differences in depuration rates of different viruses from oysters (Drouaz et al., 2015; Loisy et al., 2005; Love et al., 2010; McLeod et al., 2009a; Nappier et al., 2008; Neish, 2013; Provost et al., 2011; Ueki et al., 2007). There are several potential reasons that may account for these differences and the prolonged retention in oysters of some viruses such as NoV and HAV.

Viral localisation studies have demonstrated the presence of viruses, including NoV and HAV, in the lumen and epithelium of the digestive tract tissue (stomach, intestine and digestive diverticula), in connective tissue, and in phagocytic blood cells of oysters (haemocytes) (Fisher, 1986; Fries and Tripp, 1970; Le Guyader et al., 2006a; McLeod et al., 2009b; Provost et al., 2011; Romalde et al., 1994). Further research has demonstrated that NoV binds to certain histo blood group like antigens within oyster tissues: GI and GII NoV bind to group A like antigens in the digestive tract, which is considered to facilitate their accumulation and retention (Maalouf et al. 2010b, 2011). This specific binding may inhibit entry of NoV into the digestive system and thus protect it from degradation through the digestive process. Viruses that do not bind to these specific ligands may therefore potentially be more susceptible to elimination from oysters due to their easy entry into the digestive system, and subsequent acidic digestion within haemocytes and/or excretion through the lumen of the digestive tract.

Oyster haemocytes contain acidic vesicles which aid in the digestion of small food particles that are phagocytosed, therefore haemocytes have low pH. Provost et al. (2011) hypothesised that for viruses to persist within shellfish and haemocytes, they must be resistant to acidic digestion within haemocytes. Consistent with this hypothesis the authors demonstrated that more acid tolerant viruses persisted for longer periods in *C. virginica* (HAV>MNV>PV>FCV). Thus differences in acid tolerance between viruses may also, at least partially, account for variations in the persistence of different viruses within oysters.

5.5 Summary of factors that may influence depuration efficiency of norovirus

A variety of factors may impact the rate at which viruses are eliminated from oysters during depuration, such factors include:

- oyster species
- temperature
- salinity
- food availability (plankton)
- contamination level and length of the contamination period.

Modulation of temperature, salinity, and food availability are known to lead to changes in the filtration and clearance rates (the volume of seawater that is cleared of particles in a certain time) of bivalves. Changes in filtration rate and the consequential effects that this has on the oyster digestion rate and enzymatic activity, is likely to affect the capacity of shellfish to inactivate and eliminate viruses and likely explains changes in the depuration rate of viruses.

5.5.1 Oyster species

Few studies have directly compared the depuration rate of viruses in different oyster species, with studies generally focusing on just one species.

Nappier et al. (2008) compared uptake and retention of FRNA bacteriophage, HAV, MNV, PV and NoV in two species of oysters (*C. virginica* and *C. ariakensis*) that were depurated for a period of one

month at 20 - 23°C in separate tanks. The depuration system was a flow through system in which seawater was re-circulated back to the tanks following filtration. Oysters were fed weekly during the depuration period. *C. ariakensis* was found to be 8.4 and 11.4 times more likely to harbour NoV and HAV than *C. virginica* during depuration. Similar results were obtained when the two species of oysters were accumulated and depurated in the same tank system, with NoV remaining for 29 days in *C. ariakensis,* and 14 days in *C. virginica* (Nappier et al., 2010). These studies demonstrate that different oyster species do have varying capacity to accumulate and depurate viruses.

To our knowledge, no studies have been undertaken directly comparing the depuration rates of NoV and HAV in the two oyster species commonly found in the UK, *O. edulis* and *C. gigas*.

5.5.2 Temperature

Pacific oysters have been reported to increase clearance/filtration rates as temperatures rise from 5 to 20°C, but further increases from 20 to 32°C resulted in declining rates. With regards to native oysters (*O. edulis*), which are also commercially produced in the UK, clearance rates are reported to increase up to 30°C (Haure et al., 1998).

Consistent with this, several studies have demonstrated that higher seawater temperatures increase the rate at which viruses are depurated from oysters. It has been demonstrated that FRNA bacteriophage depurates from Pacific oysters more rapidly at higher temperatures; at 18°C levels of phage were reduced to 2% of the initial contamination level, but at 9°C, 40% of the phage remained. Similarly, Neish et al. (2013) found that a temperature of 16°C increased the depuration rate of phage when compared to a temperature of 8°C. NoV depuration was also enhanced at 16°C compared to 8°C, however the NoV reduction was still very small over 14 days (<0.5 log). Choi et al. (2016) demonstrated the persistence of NoV for six weeks in *C. virginica* depurated at 7 and 15°C, whereas NoV was only detected for four weeks in oysters held at 25°C. Love et al. (2010) also found that increased temperatures resulted in higher depuration rates for HAV, FRNA bacteriophage and poliovirus from *C. virginica.*

5.5.3 Salinity

Oysters are euryhaline and can thrive in a wide range of salinities. The salinity optima for *C. gigas* and *C. virginica* are in the range 23 - 25 ppt and 14 - 28 ppt respectively (reviewed in Gosling (2003)). The feeding rate of *O. edulis* was noted to decline at salinities above 28 ppt, and ceased below 16 ppt.

While the physiology of oysters is clearly impacted by salinity, the results of studies to date on the impact of variable salinity on viral depuration rates are not clear. Love et al. (2010) investigated the depuration of FRNA bacteriophage (MS2), PV and HAV from *C. virginica* under variable salinity (8, 18 and 28 ppt). The depuration rate of FRNA bacteriophage was found to be higher at 18 ppt than at 8 ppt, however the depuration rate of HAV and PV did not change with salinity. Nappier et al. (2008) found that salinity (8, 12 and 20ppt) did not impact virus accumulation and retention in *C. ariakensis*, but *C. virginica* was salinity dependent, with NoV and HAV not being efficiently bioaccumulated at 8 and 20 ppt, compared to 18 ppt. Love et al. (2010) and Nappier et al. (2008) both recorded mortality of *C. virginica* at 8 ppt, which corresponds with the salinity tolerance of that species.

5.5.4 Food availability

The filtration and clearance rates of oysters are known to increase with food availability, but then decrease with further rises in food concentration (Barille et al., 1997; Pascoe et al., 2009; Riisgard et al., 2003; Strohmeier et al., 2009).

Commercial depuration is normally conducted in the absence of added food, although some processes may include plankton that naturally occurs in untreated seawater. Several studies have investigated the impact of presenting oysters with a phytoplankton food source during depuration. The impact of feeding on the efficiency of NoV, TV and MgV depuration from Pacific oysters was investigated, no significant difference (P=0.25) in NoV concentrations between oysters that were fed phytoplankton (4.6×10^3 copies/g) or starved (7.6×10^3 copies/g) were observed after eight days depuration. Similarly, no difference was observed for MgV between oysters that were fed or starved, but a small difference (approximately 1 log) in TV concentration was observed (Drouaz et al., 2015).

The persistence of HAV in *C. virginica* was studied over a six-week depuration period in which the oysters were maintained at a relatively high temperature of 18°C in standard depuration conditions for the USA, with UV disinfection (Kingsley and Richards, 2003). Several groups of oysters were fed with microalgae daily phytoplankton daily (*Isochrysis* and *Tetraselmis*) to evaluate the impact of feeding on depuration. Infectious HAV (as determined by cell culture) was detected following three weeks of depuration with feeding. The presence or absence of food did not appear to affect viral persistence as determined by PCR, with HAV detected for six weeks regardless of feeding. Similarly, Love et al. (2010) found that feeding *C. virginica* with microalgae (*Isochrysis*) had no impact on the depuration of HAV, FRNA bacteriophage or PV.

6 New Approaches for Viral Depuration

Depuration was initially developed to reduce and control bacterial pathogens that were responsible for shellfish-associated outbreaks of cholera and typhoid fever. Thus, for decades the development and validation of the depuration process has focused on bacterial pathogens. It has become increasingly apparent that the traditional process does not adequately eliminate certain viruses like NoV and HAV from shellfish, and this poses a major food safety challenge. The on-going nature of viral illness outbreaks attributed to shellfish consumption, including those that have been depurated, highlights the need for improved water quality throughout the world, but also for new post-harvest treatment processes that would ensure the virological safety of shellfish. This section is focused on new potential approaches to viral depuration that may hold promise for the future.

6.1 Traditional depuration process

The traditional depuration strategy relies on the natural filter feeding process and excretion activities of bivalves to eliminate pathogenic bacteria from their digestive tracts through normal excretion when they are placed in clean seawater in conditions which allow the shellfish to function normally. As discussed, numerous modifications to the standard depuration process have been trialled, including various time, temperature and salinity regimes, feeding shellfish during the depuration process, and different disinfection approaches (e.g. UV, ozone, chlorine, iodophors). Whilst some of these modifications result in slight improvements in viral depuration rates, they have not resulted in significant reductions of the two viruses that are the predominant cause of illness outbreaks – NoV and HAV. The overwhelming finding is that the depuration process does not sufficiently eliminate NoV and HAV, with many studies showing no, or very low levels of viral reduction (Section 5.2). Given the success of depuration in reducing typhoid and cholera outbreaks, it is clear that the requirements for elimination of faecal indicator bacteria (i.e. faecal coliforms and/or *E. coli*) are significantly different to those required for viral elimination.

While the requirements for efficient bacterial and viral depuration are clearly different, there is some commonality in the initial stages of depuration. Viral depuration has been described by several authors as being 'two phase', with elimination in the first few days being more rapid than subsequent days (Love et al., 2010; Polo et al., 2014b; Polo et al., 2015; Provost et al., 2011). The first rapid phase of viral depuration is likely related to extracellular digestion and purging of the digestive tract; this process is governed by physiological traits related to the shellfish species involved which are common to both bacterial and viral depuration, including the filtration and clearance rate of the species, the digestion rate, and enzymatic activity. In this sense, the optimisation of the physiological state of the shellfish and the adjustment of the different parameters previously named (i.e. temperature, salinity and time) for each shellfish species are necessary and important to optimise reductions in viral contamination. However, the persistence of a proportion of the viral load in shellfish (the second slower phase of elimination), highlights that other properties are at play. As discussed (Section 4.3), this likely relates to the ability of some viruses and virus strains to bind to molecular receptors/ligands (HBGAs) that are present on shellfish gastrointestinal cells, and penetrate into non-conductive parts of the digestive tissue, connective tissue and other organs. This particular issue regarding viral binding represents a barrier to enhancing viral depuration and is a key point for consideration in the development of future depuration processes (Le Guyader et al., 2012; Maalouf et al., 2011; Nappier et al., 2008; Polo et al., 2014a; Polo et al., 2014b; Provost et al., 2011).

6.2 Potential new approaches

6.2.1 Enzymatic pre-treatment

Considering the specific binding of NoV to HBGAs present in oyster tissues, an approach that may have potential to enhance depuration rates is the application of certain enzymes which are known to

degrade the ligands involved. Such enzymes could be diluted in seawater and the oysters immersed for a short period of time prior to the depuration step, potentially leading to the degradation of the ligands, and enhanced release of NoV from the oyster tissues during the standard depuration process. This approach is discussed in detail in Part 2 of this report, which describes the results of pilot experiments to evaluate the efficacy of the enzymatic pre-treatment approach.

6.2.2 Bacteria

Another avenue that may be worthy of further research is the potential for particular bacteria to produce active agents/compounds that have antiviral activity, and/or induce a physiological or immune response by the shellfish that facilitates viral elimination. Various bacteriocins with antiviral activity have already been reported against several viruses, including herpes simplex type 1 (Todorov et al., 2010), influenza virus (Serkedjieva et al., 2000) and New Castle Disease Virus (Saeed et al., 2007). Lange-Starke et al. (2014) also reported a 1.25 log reduction of MNV when a lactic acid bacteria was applied, however the antiviral metabolite could not be identified.

Recently, it has been reported that a bacteria was isolated from the mussel *Mytilus galloprovincialis* and identified as *Enterococcus hirae* (designated as 3M21). The bacteria showed an antibacterial activity against *Listeria monocytogenes*, *Listeria innocua* and *Enterococcus faecalis* (Fajardo et al., 2014). In the same publication the authors reported that the bacteria showed antiviral activity against HAV and MNV-1. The active substance, which was proteinaceous in nature, has been successfully microencapsulated in alginate and is reported to be effectively ingested by oysters (Prado-Alvarez et al., 2015; Darmody et al., 2014). The efficacy of the active substance in reducing HAV and NoV titres during depuration or other post-harvest treatments has not been reported.

6.2.3 Microencapsulation

A key issue with regards to the efficacy of enzymes or bacteria in cleaving the ligand-virus specific bonds, relates to ensuring that an adequate concentration is targeted at the sites of interest within the shellfish body. Since viruses are principally concentrated in the digestive tissue, if the introduction of certain bacteria or enzymes are to have an effect it would be necessary to ensure that bacteria survive and reach the digestive gland, and that the enzymes are present in sufficient quantities at the site of interest. Additionally, chlorine, ozone and UV are all commonly used in depuration systems, and may act to reduce the viability of any bacteria.

Microencapsulation represents one possible avenue for ensuring that enzymes or bacteria applied during the depuration process reach the sites of interest in the shellfish digestive tissue. Microencapsulation techniques have been used widely in the aquaculture sector and were initially focused on overcoming marine larval feeding problems. However, their use has evolved to assist in the delivery of probiotic bacteria and bacterial substances, immunostimulants, vaccines and enzymes to target species (Polk et al. 1994; Skjermo and Vadstein 1999; Rosas-Ledesma et al. 2012; Darmody et al., 2014; Borgogna et al. 2011). Three main polymers have been broadly used for microencapsulation in the human and aquaculture sectors: chitosan, alginate and PLGA (Poly Lactic-co-Glycolic Acid) (Borgogna et al. 2011; Plant and LaPatra 2011; Luzardo-Alvarez et al., 2010; Behera et al., 2010).

While the use of probiotic bacteria, bacteriocins and inmunostimulants in encapsulated form is gaining in popularity in the aquaculture industry for targeting specific bacterial and protozoan pathogens (Martínez-Cruz et al., 2012; Darmody et al., 2014; Prado-Alvarez et al., 2015; Fajardo et al., 2014), its application in viral shellfish depuration is practically non-existent. Darmody et al. (2014) demonstrated the efficacy of delivering fluorescent particles encapsulated in alginate to target oyster

tissues. The study revealed the presence of fluorescent microbeads within the gills, digestive tubules, connective tissue and haemocytes. Similar results were also reported after the oral administration of alginate microcapsules containing inmunoestimulants in *Ostrea edulis* against the protozoan parasite *Bonamia ostreae* (Prado-Alvarez et al., 2015).

The successful ingestion of alginate microbeads by the oysters, their absorption across digestive epithelium and the release of their contents into surrounding tissues such as connective tissues, and into haemocytes (a potential virus repository), suggest that microencapsulation could represent a viable tool for the transport of antiviral substances directly to these areas.

7 Summary of Data Gaps and Limitations

Several information gaps and limitations have been identified through the literature review and are summarised below:

1. A multitude of studies have been undertaken to investigate the persistence of NoV in oysters following short term and long term depuration and relaying. These have been conducted using RT-PCR methods, as no practical culture method exists for NoV at this time. Some information on the infectivity of NoV following depuration can be inferred from illness outbreaks following depuration, a lack of illness following relaying, and from studies using culturable NoV surrogate viruses. However, the rates at which infectivity of NoV in oysters declines following depuration and relaying, under different depuration regimes (i.e. variable temperature, salinity, disinfection regimes etc), is not known.

2. Until recent times, the quantitative approach used in many depuration studies (particularly historical older research) may not have been appropriate. This is due to the lack of viral reference standards to enable accurate quantification and inappropriate sampling plans which didn't account for variation in uptake between oysters. Further studies using the standard ISO method for quantitation or new technologies, such as digital PCR, may improve our understanding of depuration rates and enable direct comparisons to be made between studies.

3. NoV persists for long periods of time in oysters due to specific binding to ligands within the oyster digestive tract, and possibly because of its high resistance to acidic conditions that are experienced within the oyster digestive system. This review demonstrates that HAV is also retained for long periods of time within oysters. The biological basis of why HAV is retained in oysters for a long time is less well understood than NoV, with no information currently available on the potential existence of ligands within oysters that HAV may bind to.

4. Numerous illness outbreaks of NoV and HAV have occurred from the consumption of depurated oysters (Table 1.3). Examination of the conditions used for depuration in these outbreaks could provide inferential information on the effectiveness of certain depuration processes. However, for most outbreaks information on the depuration conditions used is not recorded, thus it is difficult to evaluate the efficacy of the processes.

5. Many studies have investigated the impact of depuration process parameters such as temperature, time, salinity, and the feeding and disinfection approach, through modulation of one factor at a time. Only a few studies however, have sought to optimise the physiology of Pacific oysters and use a combination of conditions that promote optimal clearance rates in *C. gigas*. If further studies were conducted, care would need to be taken that conditions do not favour proliferation of potentially harmful bacteria such as *Vibrio* spp.

6. The relative rates of NoV and HAV depuration from *O. edulis* and *C. gigas* (the oyster species of relevance to the UK) have not been determined.

7. Potential differences in depuration rate for oysters that have bioaccumulated NoV in the natural environment, potentially over extended periods of time, vs. oysters that have been artificially contaminated in laboratory uptake work, is not well understood.

8 Conclusions and Recommendations

The introduction of depuration in the late 1800s was highly successful in reducing outbreaks of typhoid and cholera, however depuration has not been successful in reducing outbreaks of viral related gastroenteritis and hepatitis. There have been fifteen published illness outbreaks of NoV and HAV clearly linked to oysters that have been through standard depuration processes in a variety of countries. The oysters implicated in the outbreaks were subjected to differing depuration processes, with a range of temperatures (e.g. 8 - 12°C in France, 18°C in Australia), timeframes (ranging from 36 h to 15 days), salinities, and disinfection approaches used (e.g. UV, aeration). This information infers that NoV is resistant to depuration across a broad range of operational parameters. For six of the published outbreaks the concentration of NoV following depuration was reported, with concentrations between 1.7×10^2 and 8×10^3 NoV genome copies/g, far in excess of the infectious dose which is estimated to be as low as 10 viral particles.

While illnesses continue to occur following depuration, relaying appears to be a more successful approach, with several papers reporting significant reductions of NoV following the relay of oysters into clean open seawater for periods between 17 days and four weeks. Importantly, no illnesses were reported to be associated with relayed product, despite trace levels of NoV being detected following relaying (around the LoD of 100 genome copies/g).

A variety of factors (i.e. temperature, salinity, time) have been found to influence the rate of bacterial depuration, this largely relates to optimising depuration conditions to suit the physiology of oysters and maximise clearance rates and digestion. Optimising these environmental parameters also improves the depuration rates for most surrogate viruses (such as FRNA bacteriophage, MNV etc), but has only resulted in small (or no) improvements for NoV and HAV. This is likely related to some differences in the basic biology of the interactions between NoV and oysters, and surrogate viruses/bacteria and oysters.

Many studies have noted that depuration is a two phase process, with an initial rapid decline in contaminant level, followed by a protracted phase of slow decline. The initial rapid phase is considered to be related to extracellular digestion and purging of the digestive tract, it is thus intimately related to the physiological status of the oysters, including their clearance/filtration rate and digestion rate. Thus optimising depuration conditions such as temperature and salinity should improve viral depuration rates in the first phase of reduction – which may account for the small improvements in NoV elimination noted in studies to date. However, the retention of NoV and HAV in oysters over a long period of time during the second slow phase of elimination demonstrates that other factors are also at play for these particular contaminants (in contrast to bacteria and other surrogate viruses which are more readily excreted).

Recent publications by Le Guyader et al. (2012) and Provost et al. (2011) provide insights into the main factors that govern NoV persistence in oysters, which clearly involves a special relationship. This relationship includes the binding of GI and GII NoV to an HBGA A-like ligand present in the gut of oysters, this is considered to facilitate accumulation and retention of the virus. GII NoVs have been shown to be less well accumulated by oysters than GI NoVs, this may be related to the binding of GII NoV to sialic acid residues in the gills, which is hypothesised to lead to NoV elimination. Further, it has been demonstrated that haemocytes (key sites of intracellular digestion in oysters) are repositories of viruses in oyster tissues, thus it is considered that the high acid resistance of NoV and HAV (unlike most surrogate viruses and bacteria) may also partially account for their persistence. It is likely that these special factors that govern NoV (and HAV) retention in oysters are not influenced by basic changes in the physiology of oysters that arise due to optimising operational parameters such as temperature or feeding.

The difference between the way in which NoV (and HAV) interacts with oysters, compared to other surrogate viruses, is highlighted by the findings of this review which shows that NoV and HAV are retained for longer periods of time than a variety of surrogate viruses (including FRNA bacteriophage, FCV, MNV, PV, MgV and TV) in all comparative elimination studies to date. A comparison of the days required to achieve a 1 log reduction in virus concentration across studies to date (excluding those which show no reduction) shows that NoV takes an average of 19 days to reduce in concentration by 1 log, HAV takes 12 days, and surrogate viruses take 7.5 days.

There was no reduction of NoV in oysters noted in 50% of the studies published on NoV elimination, for those in which a reduction was demonstrated it took between 9 and 45.5 days to reduce levels by 1 log. For HAV, the number of days estimated to achieve a 1 log reduction was between 7 and 16.1 days, with two of the five published experiments showing no loss of HAV (Table 1.5). The timeframes required to achieve a 1 log reduction are clearly much longer than that routinely used for depuration, which in the UK is recommended to be 42 h. Further, illness outbreaks commonly involve NoV concentrations of around 10^3 viral copies/g. Thus a one log reduction is unlikely to be sufficient to protect public health, and further time would be required to reduce levels sufficiently. In contrast, relaying oysters to areas with clean seawater over a four-week period appears to be successful in reducing NoV levels to around the LoD (100 genome/copies), and no illnesses have been reported following the consumption of relayed oysters to date.

In light of the special relationship between NoV (and HAV) and oysters, and the limited success of studies which have focused on optimising operational parameters of the depuration process (i.e. temperature, salinity, feeding etc), it is suggested that the following topics be given priority when considering future research to support the production of virologically safe oysters:

- In a detailed review of depuration undertaken by Richards in 1988 he comments that "depuration was not intended for grossly polluted shellfish or for shellfish harvested from grossly polluted waters". This comment remains as valid today as it was in 1988. Bearing this in mind, the major focus should be placed on improvements in water quality to avoid NoV and HAV contamination of shellfish at source. To this end, further collaboration between water companies, local authorities and the shellfish industry should be prioritised to improve wastewater treatment, and processes governing discharges and communication of these to all affected parties.
- Improvement of our understanding of the special virus-oyster relationship and binding interactions, particularly for viruses of high concern such as HAV, for which less information is known.
- Investigations into post-harvest interventions that exploit the mechanisms by which NoV is retained (binding to HBGA a-like antigens) and potentially eliminated (binding to sialic acid ligands) from oysters.
- Limited information currently exists regarding the time over which NoV infectivity is retained in oysters during depuration and relaying. Some inferential information is available from HAV studies (which have involved culture methods), and from epidemiological observations following the consumption of depurated and relayed oysters. However, more direct information on the infectivity of NoV over the course of purification would be informative for risk management purposes. Recent advances in the US have resulted in a cell culture method for NoV being developed. This is not likely to be amenable to routine use for the analysis of shellfish, however, it may be useful to have access to this capacity through collaboration on limited studies that investigate viral infectivity during depuration/relaying.

Part Two: Pilot Laboratory Study: Can Enzymatic Pre-treatment Increase Depuration Efficiency?

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1 Introduction

Norovirus (NoV) persists in shellfish tissues for longer periods of time than faecal bacteria. Recent studies have demonstrated selective accumulation and retention of different NoV strains in oysters via specific binding to carbohydrate ligands known as histo blood group antigens (HBGAs). These ligands are mainly localised in the digestive tissues (DT) of the oyster (but also in the gills and labial palps) and are similar to receptors involved in human NoV infections (Le Guyader et al., 2006a; Le Guyader et al., 2012; McLeod et al., 2009a, 2009b; Tian et al., 2006). GI and GII NoV strains are known to bind to Type-A HBGAs, while GII strains also bind to sialic acid residues. Thus, shellfish use HBGAs to actively accumulate particular virus types, and even strains. Consistent with this, molecular epidemiology of shellfish related outbreaks show a high prevalence of some NoV GI strains, compared to general outbreaks in which GII strains dominate (Yu et al., 2015). The specific binding of NoV to HBGAs probably explains viral persistence and the failure of different depuration strategies tested to date (Polo et al., 2014a; Richards et al., 2010).

Certain enzymes have the ability to degrade HBGAs, which are complex carbohydrates, through the cleavage of certain bonds in the carbohydrate structure. Thus, application of particular enzymes to NoV contaminated oysters could result in destruction of the HBGAs and destabilisation of the specific NoV-HBGA linkages. This component of the project describes a series of pilot experiments conducted to investigate the hypothesis that an enzymatic pre-treatment step prior to depuration will damage the HBGAs in oysters, leading to the release of NoV particles and enhanced depuration rates.

1.1 Identification and selection of compounds

The composition and structure of HBGAs is important to consider when selecting the most promising enzymes to trial. The HBGAs are carbohydrates that contain structurally related saccharide moieties. Type-A antigens (GalNAcα1-3(Fuc-α1-2)Gal) are generated by transfer of GalNAc and Gal residues to a core structure of (Galβ1-3GlcNAcβ) in type 1A antigens, or (Galβ1-4GlcNAcβ) in type 2A antigens (Shirato, 2011). Sialic acid (SA) is a generic term for the N- or O-substituted derivatives of neuraminic acid, a monosaccharide with a nine-carbon backbone. The most common member of this group is the N-acetylneuraminic acid (Neu5Ac) (Varki, 1992). A variety of compounds that have the ability to degrade Type-A and SA antigens within oyster tissues has been identified from the literature; these are presented in Appendix 3 (Davies et al., 2005; Sova et al., 2013; Bakunina et al., 2012; Feng et al., 2013).

The first group of compounds with the potential to act against the HBGAs of concern are the proteases. Proteases are extensively used for the modification of red blood cell membranes and are frequently used in blood group serology. They cleave proteins at defined sites along peptide sequences. The broad activity and low specificity of proteases (even in the presence of various salts) makes them promising candidates for targeting the protein structures involved in NoV binding. Different types of proteases are able to be commercially sourced, including those of animal (trypsine, lipase) and plant origin (proteinase K, papain) (Appendix 3).

The second group of promising compounds are the glycosidases (glycosyl hydrolases). Glycosidases catalyse the breakage of glycosidic bonds in complex sugars. Depending on the cleavage site,

glycosidases can be classified as endo- (non-terminal residues) or exo-glycosidases (terminal residues). Although these compounds are more specific than proteases, they have great potential to destroy the carbohydrate moieties that are integral components of the HBGAs. Of particular interest are α-amylase, which acts upon α(1,4)-D-glucosidic linkages, and β-galactosidase hydrolyses β-glycosidic bonds, which may favour the hydrolysis of type-A HBGAs (Appendix 3).

Compounds that degrade the sialyl groups that are present in some HBGAs may favour the release of NoV GII strains, which bind to SA residues. Sodium periodate ($NaIO_4$) may show promise in this regard, as it has an oxidising effect on glycans that contain sialyl groups and is non-enzymatic and therefore more stable in seawater than proteases and glycosidases.

Eight of these compounds were selected for use in the pilot experiments, including $NaIO_4$, two types of proteinase K (PK), α-amylase, β-galactosidase, lipase, trypsin and papain. The rationale for selecting these compounds is: (1) their potential to disrupt the NoV-HBGA linkage; (2) the compounds selected represent each of the major groups identified as being potentially effective (animal and plant proteases, glycosidases and $NaIO_4$) (blue shading, Appendix 3); and (3) the compounds can be sourced in sufficient quantities for the experiments.

1.2 Food safety considerations

A consideration with respect to the potential use of enzymes/bacteria during the depuration process is whether these substances are safe for human consumption if residual levels remain in market-ready oysters. To fully evaluate the safety aspects, it is necessary to undertake a risk assessment to evaluate the toxicology of the compounds involved, the quantities that may be present in oysters following treatment, and the potential for acute and/or chronic impacts in consumers. Such an assessment is not possible at this time, as antiviral substances that are effective would first need to be identified, and the parameters of their use in the depuration process defined. However, it is noted that several of the substances that may be effective are already used in the food industry as processing aids and additives, which indicates that their application during the depuration process may not necessarily pose an undue risk to consumers. The following provides an overview of some of the compounds that may potentially degrade NoV ligands and their current application in the food processing industry.

Serine proteases (Enzyme Commission number: EC 3.4.21), such as trypsin, α-chymotrypsine and PK, are the largest group of proteases (Hedstrom, 2002). They are extensively used in the food and animal feed industries due to their optimal activity at neutral or alkaline pH (pH 7 - 11). They are used to produce protein hydrolysates from whey, casein, soy, keratinous materials, as well as scraps from meat and fish processing. They are also used for the development of flavour during ripening of dairy products (Dalev, 1994; Wilkinson and Kilcawley, 2005).

Sulfhydryl proteases, also known as thiol or cysteine proteases (EC 3.4.22) (e.g. papain, chymopapain, bromelain, ficin), tend to be optimally active at neutral pH (pH 6 - 7.5) and are relatively heat stable, which accounts for their use as meat tenderisers. These proteases may be applied in various ways to achieve meat tenderisation e.g. blending, dipping, dusting, soaking, spraying, injection and vascular pumping (Etherington and Bardsley, 1991). Sulfhydryl proteases are also used in the brewing industry to control haziness and improve the clarity of beer, and in the baking industry to improve the elasticity and firmness of dough through the modification of gluten (Mathewson, 1998).

Metalloproteases (EC 3.4.24), like pronase E or O-sialoglycoprotease, are characterised by having metal ions in their active sites. Some of the industrial applications of metalloenzymes include the synthesis of peptides for use as low calorie sweeteners. Pronase E is also used, alone or in combination with other proteases (protease cocktails), for the production of food protein

hydrolysates, flavour-enhancing peptides, and for accelerating the ripening of dry sausages (Fernandez *et al*., 2000).

Glycosidases (EC 3.2.1) are carbohydrate processing enzymes (or carbohydrases) that catalyse the hydrolysis of glycosidic bonds to liberate monosaccharides and oligosaccharides. α-amylases are the most common carbohydrases used in food processing; they are used by the starch, alcoholic beverages and sugar industries. Lactases or β-galactosidases are also common and are used to catalyse the breakdown of the milk sugar, lactose, into its constituent monosaccharides, galactose and glucose. β-galactosidases are widely used in the dairy industry to produce fermented milks, ice-cream, milk drinks and lactose-reduced milk (Simpson *et al*., 2012). O-glycosidases are used for flavour enhancement in beverages such as fruit juices and particularly in wine processing (Sarry and Gunata, 2004).

Lipases, or triacylglycerol acylhydrolases (EC 3.1.1.3), are hydrolytic enzymes that catalyse the breakdown of ester bonds in biomolecules such as triglycerides, phospholipids, cholesterol esters and vitamin esters. Lipases are water-soluble proteins (Wang and Hartsuck, 1993). Lipases are used in the manufacture of dairy products e.g. milk, cheese and butter to facilitate the ripening process and develop flavouring. They are also used to improve the flavour or aroma of bakery foods and beverages and for quality improvement of mayonnaises, dressings and whippings (Schmidt and Verger, 1998; Sharma *et al*., 2001).

1.3 Regulatory considerations

Substances that are added to foods are classified differently in the EU, depending on their purpose and the way they are used in the manufacturing process.

Food ingredients are generally considered to be substances that are used in the manufacture or preparation of a food and that are still present in the finished product, even in an altered form.

Food additives are substances that are not normally consumed as food or used as a characteristic ingredient of food, but are added to the food intentionally for a technological purpose. Regulation (EC) No. 1333/2008 defines a food additive and lists the approved food additives and sets condition of use.

Processing aids are substances that are not consumed as foods, but are used in the processing of raw materials, foods or their ingredients, for a technological purpose, and residues of the substance (or derivatives) may be found in the final product. Any residues should be safe and not have a technological effect in the final product. Processing aids are defined in the food additive legislation, but this legislation does not control their use.

Food enzymes are defined as substances capable of catalyzing a specific biochemical reaction and are added to food for a technological purpose. The rules for the use of enzymes in the EU is covered under Regulation (EC) No. 1332/2008 and this covers both enzymes used as food ingredients and as processing aids. The European Commission is working towards the establishment of a positive list of food enzymes.

An assessment would need to be made as to the classification of a substance applied during depuration under EU law. For food enzymes it would be necessary to ensure that data required for risk assessment and risk management is gathered (consistent with the requirements of Regulation (EC) No. 234/2011). This would enable the European Food Safety Authority (EFSA) to assess the safety of the enzyme and for the European Commission (and Member States) to form an opinion as to

whether the enzyme in question should be included on the eventual EU approved list for food enzymes.

1.4 Objectives and approach

The main objective of the pilot experiments is to evaluate if the treatment of NoV-contaminated oysters with specific compounds prior to depuration improves NoV reduction rates. To investigate the efficacy of various compounds, two different experimental approaches were pursued.

Firstly, ten bioaccumulation and depuration trials were performed using oysters contaminated with either NoV GII, or a mixture of NoV GI and GII. Contaminated oysters were immersed for one or two hours in each of the eight different bioactive compounds as a pre-treatment step prior to depuration. Two concentrations of each compound were evaluated, using three different seawater temperatures.

Secondly, due to the time intensive nature of the bioaccumulation and depuration experiments, an *in vitro* approach was developed whereby the DT of NoV contaminated oysters were treated with the bioactive compounds in 24-well cell culture plates. If compounds that are effective in destabilising the HBGAs are applied, there is a reduction in the NoV levels in the DT (or an increase in Ct value[2]). This *in vitro* assay enabled additional screening of the candidate compounds.

[2]The relationship between cycle threshold values (Ct) obtained using real time RT-PCR and the concentration of viral particles is an inverse relationship – that is - the higher the Ct value, the lower the concentration of viral genomes present.

2 Materials and Methods

2.1 Bioaccumulation

Pacific oysters (*Crassostrea gigas*) were sourced from a single supplier to minimise natural variation in uptake rates between oysters. The oysters were first acclimatised to being maintained in tanks with natural seawater and continuous aeration for a period of four hours until all the oysters showed correct filtering activity (open shells) and were then subjected to a bioaccumulation step for 24 hours to contaminate them with NoV.

A total of ten bioaccumulation trials were performed, nine using NoV GII.3 and one using NoV GI.1 and GII.3 as a mixture. These strains were selected as they have been previously used for ligand characterisation and tissue distribution studies; it is known that they accumulate efficiently and can therefore, be considered as a 'worst' case (compared to Nov GII.4, for example). A stool suspension was prepared at the beginning of the study and aliquots of 10 ml were frozen allowing for good reproducibility of the bioaccumulation process. Each bioaccumulation trial was performed with approximately 50 oysters. The oysters were uniformly dispersed in a monolayer in tanks along with 10 litres of natural seawater. NoV (5×10^8 RNA copies) was then added to the tanks containing the oysters for a period of 24 h. Bioaccumulation of the oysters was undertaken in seawater maintained at ambient room temperature (around 15 ± 3°C). The NoV concentration after bioaccumulation (and prior to treatment and depuration) was determined for each trial to verify viral uptake by the oysters (see Section 3.2 and Tables 2.1 and 2.2 for results). Contaminated oysters were relocated to tanks with clean seawater and washed to remove intra-valvular fluid and associated viruses that had not been internalised.

Oysters from each trial were then divided into groups of eight to ten individuals, resulting in five to seven groups per trial (with the exception of trial 4, which is discussed further below). Each group of eight to ten individuals was treated as a single pooled sample, using this 'pooled sample' approach assisted in overcoming potential issues relating to variation in NoV uptake by individual oysters. For each trial, one sample (i.e. one group of 8 -10 oysters) acted as a control sample and was subjected to depuration without exposure to the bioactive compounds prior to purification. The viral content of control samples is shown in Tables 2.1 and 2.2. The remaining four – six samples (per trial) were subjected to treatment with the bioactive compounds, as discussed in further detail for each trial below.

2.2 Depuration trials

As noted, the efficacy of eight different compounds was evaluated (Section 1.1). Two different concentrations of each of the compounds were trialled, at three temperatures (14°C, 20°C and 27°C). The higher temperatures were used in the initial experiments to mimic, as far as possible, the optimal activity conditions of the enzymes. Latter experiments were conducted at 14°C to reflect temperatures commonly used for depuration in commercial settings in the UK, as identified in Part 1 of this report (Part 1, Section 3.2.2). All bioaccumulation and depuration trials were conducted in seawater at pH 7.5 and salinity of 35 ppt.

Treatment with each of the eight compounds was performed for either one (trials 1-6) or two hours (trials 7 - 10), followed by depuration in clean seawater for either 24 hours, 48 hours or one week at the temperatures noted above (control samples were subjected to the same depuration conditions). Depuration was performed in small tanks (around 50 litres of seawater), with aeration using filtered natural seawater (with no feeding or UV treatment). Table 2.1 gives details of the compounds and conditions tested in each of the ten depuration trials, in summary:

- Trial 1: Two concentrations of PK from *Tritirachum album* (10 and 100 mg/L; 300 and 3000 units (U), respectively) and $NaIO_4$ (1 and 10 millimolar (mM)) were tested using seawater at room temperature (around 20°C). PK at the highest concentration (100mg/L) was also tested at 27°C, in an attempt to be closer to the optimal activity of PK (37°C) within the physiological capacity of the oysters. NoV GI.1 and GII.3 was used for the bioaccumulation step. The treatment and depuration periods were 1 and 24 h respectively.
- Trial 2: Two different types of PK from *Tritirachum album* (PK in powder and PK glycerol solution) were tested. Four treatments were performed: (1) PK in powder for 1 h (100 mg/L; 3000U); (2) PK in glycerol solution for 1 h (100 mg/L; 3000U); (3) a mix of both types of PK for 1 h (200 mg/L; 6000U); and (4) a sequential treatment (200 mg/L; 6000U) involving treating the oysters for 1h with PK in powder, relocating the oysters to clean seawater for 1 h, then adding PK in glycerol solution and treating the oysters for an additional 1h. The temperature of the seawater was 27°C. The depuration period was 24 h.
- Trial 3: Two concentrations of α-amylase from porcine pancreas (300 and 3000 mg/L; 3000 and 30000U, respectively) were tested. This trial was performed at two seawater temperatures (20 and 27°C). The treatment and depuration periods were 1 and 24 h respectively.
- Trial 4: β-galactosidase (50 mU) from *Bacteroides fragilis* (expressed in *E. coli*) was trialled. Due to the special characteristics of this compound it is commercially available in very small quantities, thus this trial was carried out using very small samples numbers (five oysters). Three oysters were analysed individually to determine the initial concentration of NoV after the bioaccumulation step and one oyster was used as a control (and the NoV concentration post depuration with no treatment was determined). The remaining oyster was treated with β-galactosidase. The treatment and depuration periods were 1 and 24 h respectively.
- Trial 5: Lipase from *Candida rugosa* was tested at two concentrations (100 and 1000 mg/L) and two seawater temperatures 20 and 27°C. The treatment and depuration periods were 1 and 24 h respectively.
- Trial 6: Papain from *Carica papaya* latex (100 mg/L) was tested at two different temperatures of seawater (20 and 27°C) and trypsin from bovine pancreas (100 and 1000 mg/L) was tested at 27°C. The treatment and depuration periods were 1 and 24 h respectively.
- Trial 7: Four concentrations of $NaIO_4$ (0.05, 0.1, 0.5 and 1 mM) were tested using seawater at 14°C. The treatment time was 2 h. The depuration time was 24 h. Gill and DT were analysed.
- Trial 8: Three concentrations of PK from *Engyodontium album* (500, 1500 and 5000U) were tested using seawater at 14 °C. Two additional batches were included using PK that had been previously activated in distilled water at 37°C. The treatment time was 2 h. One of the additional batches was subjected to depuration for 48 h and the other batch was depurated for one week (in this case seawater was changed every two days). Gill and DT were analysed.
- Trial 9: Three concentrations (300, 3000 and 6000 mg/L) of α-amylase from porcine pancreas were tested. The treatment time was 2 h and the depuration period was 2 4 h. Seawater temperature was 14°C. DT were analysed.
- Trial 10: A further trial was undertaken using α-amylase from porcine pancreas with 2 h treatments. Enzyme treatment was followed by 24 h of depuration (6000 mg/L), and 48 h depuration (3000 mg/L and 6000 mg/L) at 14°C.

Table 2.1. Summary of the compounds tested and conditions used in depuration experiments

Trial	Compound	Source of compound	Concentration	Temperature of seawater (°C)	Treatment period	Depuration period	Tissues analysed
1	Proteinase K	*Tritirachum album*	300 Units 3000 Units	20 20 and 27	1 hour	24 hours	DT
	$NaIO_4$	Not applicable	1mM 10mM	20	1 hour	24 hours	DT
2	Proteinase K (powder form) Proteinase K (glycerol form) Proteinase K (powder + glycerol forms) Proteinase K (powder + glycerol forms)	*Tritirachum album*	3000 Units 3000 Units 6000 Units 6000 Units	27	1 hour 1 hour 1 hour[a] 1 hour[b]	24 hours	DT
3	α-amylase	Porcine pancreas	300 mg/L 3000 mg/L	20 and 27	1 hour	24 hours	DT
4	β-galactosidase	*Bacteroides fragilis*	50 mU	27	1 hour	24 hours	DT
5	Lipase	*Candida rugosa*	100 mg/L 1000 mg/L	20 and 27	1 hour	24 hours	DT
6	Papain	*Carica papaya* latex	100 mg/L	20 and 27	1 hour	24 hours	DT
	Trypsin	Bovine pancreas	100 mg/L 1000 mg/L	27	1 hour	24 hours	DT
7	$NaIO_4$	Not applicable	0.05, 0.1, 0.5, 1 mM	14	2 hours	24 hours	DT and gill
8	Proteinase K	*Engyodontium album*	500 Units 1500 Units 5000 Units	14[c]	2 hours	24 hours, 48 hours or 1 week	DT and gill
9	α-amylase	Porcine pancreas	300 mg/L 3000 mg/L 6000 mg/L	14	2 hours	24 hours	DT
10	α-amylase	Porcine pancreas	3000 mg/L 6000 mg/L	14	2 hours	48 hours 24 and 48 hours	DT

[a]Mix of both types of PK (PK in powder and in glycerol) for 1 h

[b]Sequential treatment (200 mg/L; 6000U) involving treating the oysters for 1h with PK in powder, relocating the oysters to clean seawater for 1h, then adding PK in glycerol solution and treating the oysters for an additional 1 h

[c]Two additional batches were included using PK that had been previous activated in distilled water at 37°C.

2.3 Evaluation of compound efficacy using an *in vitro* method

Oysters bioaccumulated NoV GII.3 as detailed in Section 2.1. The DT of the oysters was dissected and carefully chopped into small pieces and gently washed with phosphate buffered saline (PBS) to remove virus that was not firmly associated with the DT. DT pieces were then distributed into each well in a 24-well cell culture plate, to obtain a weight of 200 mg of DT/well, and each well was treated with the selected compounds for 1 h. Control wells were included and were treated only with PBS. In some *in vitro* assays, gills were also analysed in the same manner as the DT.

After the treatment period the supernatant component (ST) and DT (or gill) component were recovered and then analysed separately to determine what proportion of virus remains attached to the DT, and what proportion has been 'cleaved' and eluted into the ST. Increases in Ct values (i.e. a decrease in viral concentration) in the DT samples following treatment indicates that the compounds have cleaved the virus from the ligand. Viral recovery from the DT and ST, RNA extraction and quantification by real-time RT-PCR was carried out according to the ISO/TS 15216-1 method with minor modifications (Section 2.4). A schematic outline of the final *in vitro* process used is shown in Figure 2.1.

An initial experiment was undertaken to investigate the reproducibility and practicality of the *in vitro* method using Eppendorf tubes, or 24-well cell culture plates. This involved investigating the impact of three concentrations of $NaIO_4$ on NoV levels in triplicate samples of DT and ST using Eppendorf's and 24-well plates. Both assay formats gave reproducible results with relatively low standard deviations (SD) (Appendix 4), however the 24-well plate format was easier to perform and less time consuming and was therefore used for all experiments.

2.4 Method of analysis

Viral recovery from shellfish, RNA extraction and quantification by real-time RT-PCR (RT-qPCR) was carried out according to the ISO/TS 15216-1 method with minor modifications (Le Guyader et al., 2009). For the extraction step, mengovirus (MgV) was used as an extraction control to measure the recovery (%) of viruses from the oysters. NoV quantification was performed using real-time RT-PCR as previously described (ISO/TS15216-1), using standard curves in each amplification series. Additionally, RNA extracted from the oysters was analysed 'neat' (undiluted) and diluted (1/10) to assist in overcoming any issues relating to reaction inhibition. The extraction efficiency was checked for each extraction to avoid potential technical problems due to the use of enzymes. If required, extractions were repeated.

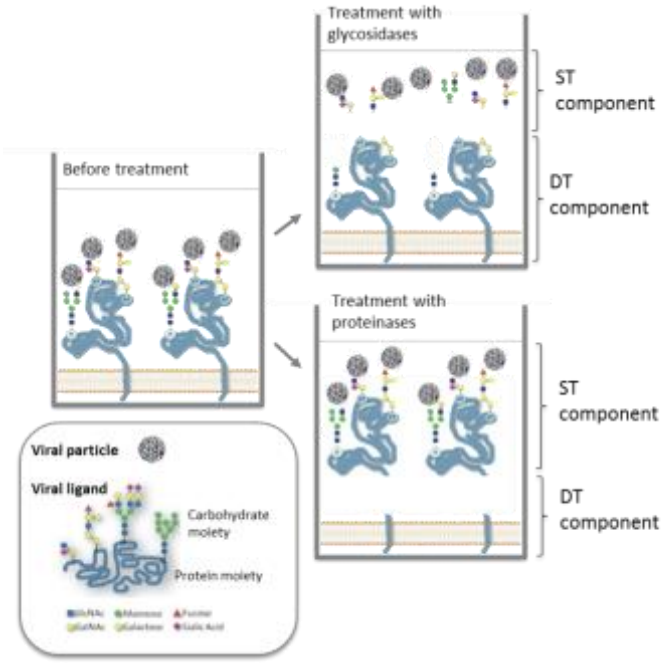

Figure 2.1: Schematic outline of *in vitro* process used. The figure shows a virtual cell-culture well containing the oyster digestive tissue (DT) and supernatant (ST) portions and associated glycoproteins (composed of a carbohydrate moiety and protein moiety) where viral particles bind. If the compound used is effective, the viral particles will be cleaved from the carbohydrate moiety (glycosidases) or from the protein moiety (proteinases).

3 Results

3.1 Quality control for RNA extraction and real-time RT-PCR

Extraction efficiency values, determined by the addition of a known amount of MV in each RNA extraction series, showed that samples were suitable for quantification and the data were consistent. Following the ISO/TS 15216-1 method recommendations, only extraction efficiency values greater than 1% were considered to be acceptable and included in the study. Notably, in a recent field study involving the analysis of 168 oyster samples, we found no relationship between samples showing acceptable but poor (1-5%) extraction efficiencies and the quantity of NoV detected, compared to those with better extraction efficiencies (>5%) (Le Mennec et al., 2016). This indicates that quantification of NoV in samples with extraction efficiencies above 1% is valid. Extraction efficiency values for DT were between 1.2% and 61.8% for NoV GII.3 and between 9.8% and 25.2% for NoV GI.1.

3.2 Virus concentration following bioaccumulation

The concentration of NoV GII.3 in oysters following bioaccumulation (but prior to enzyme treatment and depuration) in trials 1 – 6 was between 3.5 $\times 10^4$ and 5.6 $\times 10^5$ RNA c/g DT, with a geometric mean titre (GMT) of 9.8 $\times 10^4$ RNA c/g DT. For trial 1 there was 2.0 $\times$ 10^3 RNA c/g DT of NoV GI.1 present. For the experiments conducted at 14°C (trials 7 to 10), the initial NoV GII.3 levels were between 1.2 $\times 10^3$ and 1.3 $\times 10^6$ RNA c/g DT, with a GMT of 1.9 $\times 10^4$ RNA c/g DT. In these trials, gill tissue was also analysed and the initial NoV GII.3 concentrations prior to enzyme treatment and depuration were between the limit of quantification (100 RNA c/g DT) and 1.9 $\times 10^2$ RNA c/g gill.

3.3 Depuration trials

The depuration trials were conducted using three different temperatures: trials 1 to 6 were carried out from March 2015 to June 2015 at 20°C and 27°C. Trials 7 to 10 were carried out from November 2015 to March 2016 at 14 ± 1°C, this temperature was selected to more closely mimic industry practices. Compounds and conditions used in each trial are shown in Table 1, with respective results presented in Tables 2.2 (Trials 1 – 6) and 2.3 (Trials 7 – 10), and Figures 2.2 and 2.3.

For experiments conducted at 20 and 27°C, the GMT of NoV GII.3 in oysters following bioaccumulation was 9.8 x 10^4 RNA c /g of DT, and for NoV GI.1, the GMT was 2 x 10^3 RNA c/g of DT. Generally, enzymatic pre-treatment followed by depuration did not result in large decreases in NoV concentrations in the DT (Tables 2.2 and 2.3), however small reductions were observed for some compounds, particularly papain, $NaIO_4$, PK and trypsin. Figure 2.2 illustrates data presented in Table 2.2 and shows that NoV GII.3 was reduced by a further 68% in oysters that were treated with 100 mg/L papain when the seawater temperature was 20°C when compared to the control sample (only subjected to depuration). NoV was reduced by a further 56% in oysters treated with 1 mM $NaIO_4$, compared to control samples which were depurated at 20°C. Oysters treated with 100 mg/L trypsin or 100 mg/L PK showed higher reduction rates than control samples when depurated at 27°C (68% and 65% further reduction in NoV respectively).

For two trials (PK in trial 1 and amylase in trial 3) an apparent increase in NoV GII.3 levels was observed following some treatments (Table 2.2). However, the increase was small and likely due to variation in the viral content of the samples after bioaccumulation. The variability in the initial viral loads among trials might be explained by natural variation in individual physiological activity, shellfish age and the condition of each oyster. These factors can affect the filtering and uptake processes, even when experimental lab conditions are identical. These phenomena have been previously observed in different studies using a variety of viruses and shellfish species (Canzonier, 1971; Hernroth and Allard, 2007; Love et al. 2010; Ueki et al. 2007; Polo et al. 2014a, b). This variation should be considered

when interpreting the results of the reduction studies, because small decreases in NoV may be a function of variability rather than the effect of the compounds. Considering this, PK, trypsin and papain showed the largest reductions and were the most promising compounds trialled at higher temperatures; while the initial trials with $NaIO_4$ at 20°C showed reductions, further trials at 14°C exhibited no enhancement of depuration – as discussed further below.

For NoV GI.1 (Table 2.3), only two compounds (PK and $NaIO_4$) were tested (trial 1), PK did not enhance the depuration of NoV, and $NaIO_4$ at 10 mM (20°C) showed a very marginal enhancement in viral reduction (14.5 %).

Table 2.2. NoV GII.3 levels in oysters following immersion for one hour in various compounds and depuration for 24 hours at either 20 and 27°C. Control samples shown were depurated, but not subjected to treatment with the compounds.

Trial	Compound	Temp (°C)	mg/L[a]	Eff. Ext.[b] (%)	Mean Ct[d] value	RNAc/g DT[d]
1	*Initial load*[c]	20	-	*10*	*24.6*	*3.6 x 10^5*
	Control		*0*	*17*	*24.6*	*3.7 x 10^5*
	PK		10	13	23.9	5.9 x 10^5
	PK		100	14	23.7	6.4 x 10^5
	$NaIO_4$ (1mM)		214	24	25.8	1.7 x 10^5
	$NaIO_4$ (10mM)		2140	21	25.1	2.7 x 10^5
	Control	27	*0*	*10*	*27.9*	*4.4 x 10^4*
	PK		100	25	27.6	5.3 x 10^4
2	*Initial load*[c]	20	-	*9.5*	*27.3*	*6.7 x 10^4*
	Control		*0*	*21*	*26.2*	*1.3 x 10^5*
	PK		100	12	27.8	4.8 x 10^4
	PK glycerol		100	6	26.9	8.3 x 10^4
	PK mix		200	27	27.0	7.7 x 10^4
	PK sequential	27	200	7	26.4	1.1 x 10^5
3	*Initial load*[c]	20	-	*19.9*	*27.6*	*5.4 x 10^4*
	Control		*0*	*9*	*28.3*	*3.5 x 10^4*
	α-Amylase		300	24	28.8	2.6 x 10^4
	α-Amylase		3000	10	27.6	5.2 x 10^4
	Control	27	*0*	*23*	*28.4*	*3.3 x 10^4*
	α-Amylase		300	23	28.1	3.8 x 10^4
	α-Amylase		3000	22	27.7	4.9 x 10^4
4	*Initial load*[c]	27	-	*47.8*	*23.9*	*5.6 x 10^5*
	Control		*0*	*43*	*24.6*	*3.6 x 10^5*
	β-galactosidase		50 mU	50	23.9	5.7 x 10^5
5	*Initial load*[c]	20	-	*39.7*	*28.3*	*3.5 x 10^4*
	Control		*0*	*19*	*27.2*	*7.0 x 10^4*
	Lipase		100	28	26.7	9.4 x 10^4
	Lipase		1000	27	28.2	3.7 x 10^4
	Lipase		100	15	27.2	7.0 x 10^4
	Lipase	27	1000	22	27.2	7.0 x 10^4
6	*Initial load*[c]	20	-	*14.4*	*28.3*	*3.5 x 10^4*
	Control		*0*	*32*	*25.9*	*1.6 x 10^5*
	Papain		100	6	27.7	5.0 x 10^4
	Papain		100	9	27.1	7.5 x 10^4
	Trypsin		100	15	27.7	5.0 x 10^4
	Trypsin	27	1000	10	26.9	8.3 x 10^4

[a]Compounds tested and their final concentrations; [b]the extraction efficiency; [c]NoV concentration following bioaccumulation (no treatment or depuration); [d]Results (expressed as Ct values, and RNA copies/g DT) for each trial are shown. PK = proteinase K. $NaIO_4$ = sodium periodate.

Table 2.3. NoV GI.1 levels in oysters following immersion for one hour in various compounds and depuration for 24 hours at either 20 and 27°C. Control samples shown were depurated, but not subjected to treatment with the compounds.

Trial	Compound	Temp (°C)	mg/L[a]	Eff. Ext. (%)[b]	Mean Ct value[d]	RNAc/g DT[d]
1	*Initial load*[c]	20	-	*10*	*32.8*	*2.0 x 10^3*
	Control		-	*17*	*33.7*	*1.1 x 10^3*
	PK		10	13	31.7	4.1 x 10^3
	PK		100	14	32.2	2.8 x 10^3
	$NaIO_4$ (1mM)		214	24	33.7	2.0 x 10^3
	$NaIO_4$ (10mM)		2140	21	32.7	9.4 x 10^2
	Control	27	-	*10*	*33.9*	*9.8 x 10^2*
	PK		100	25	33.7	1.1 x 10^3

[a]Compounds tested and their final concentrations; [b]the extraction efficiency; [c]NoV concentration following bioaccumulation (no treatment or depuration); [d]Results (expressed as Ct values, and RNA copies/g DT) for each trial are shown. PK = proteinase K. $NaIO_4$ = sodium periodate.

To investigate the effect of the compounds when depuration is conducted at lower temperatures, reflecting those more routinely used in the UK, some experiments were repeated (trials 7 – 10) at 14°C (Table 2.4, Figure 2.3). For these experiments the GMT for NoV in oysters following bioaccumulation was 1.9 x 10^4 RNA c /g of DT and 90 RNA c/g of gills.

At 14°C, two compounds showed an enhanced reduction in viral levels in comparison to non-treated control samples: PK (500U) with 60% reduction; and α-amylase at 3000 and 6000 mg/L, with 61 and 80% reduction respectively (Figure 2.3). α-amylase at a concentration of 6000 mg/L was the compound that showed the highest level of reduction (trial 9), however this was not able to be repeated and confirmed in a subsequent experiment (trial 10), corroborating results obtained with trial 3 which showed no reduction at 20 - 27°C. $NaIO_4$ did not enhance virus removal confirming that the small decrease in NoV observed at 20 - 27°C may relate to the higher seawater temperature and an increase in the oysters physiological activity.

For trial 8 at 14°C, a sample of oysters treated with PK was maintained in clean seawater under aeration for a week to investigate if long term depuration would lead to further NoV reductions, however, no further decrease was observed (final concentration of 3.92 x 10^4 RNA c/g of DT).

As gills are one of the first organs to come into contact with contaminated seawater, the effect of two compounds (PK and $NaIO_4$) on the reduction of NoV in the gills following depuration was examined (trials 7 and 8). Only low levels of NoV were detected in the gills following bioaccumulation (Table 2.4), confounding interpretation, however it appears that neither $NaIO_4$ nor PK is effective in enhancing depuration of NoV from this tissue.

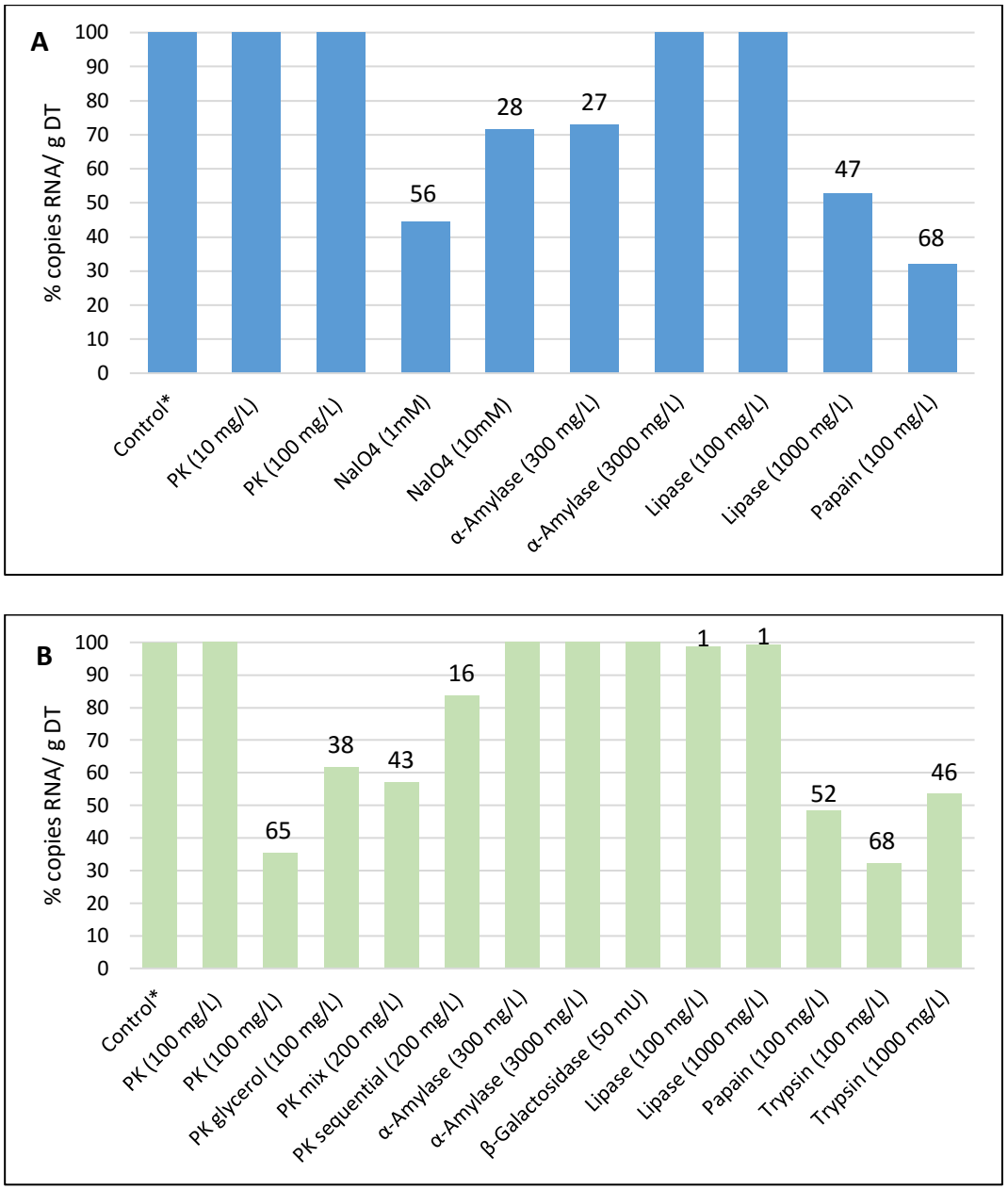

Figure 2.2: Schematic representation of NoV GII.3 removal in oyster DT following a one hour enzyme treatment step and 24 hours of depuration at 20°C (A) and 27°C (B). The bars show the amount (as a %) of NoV remaining in oysters following treatment when compared to control samples (which were not subjected to enzymatic pre-treatment) i.e. smaller bars indicate a larger reduction in NoV. Values above bars show the decrease in NoV (as a %), compared to control samples.

Table 2.4. Concentration of NoV GII.3 in oysters following treatment for two hours with various compounds and depuration at 14°C.

Digestive Tissue					
Trial	**Compound**	**mg/L[a]**	**Eff. Ext. (%)[b]**	**Mean Ct value**	**RNAc/ g DT**
	Initial load[c]		*15.0*	*20.9*	*1.33 x 10^6*
7	*Control*	-	*17*	*23.62*	*2.30 x 10^5*
	NaIO4 (0.05 mM)	10	16	21.98	6.46 x 10^5
	NaIO4 (0.1 mM)	20	12	21.51	8.83 x 10^5
	NaIO4 (0.5 mM)	100	9	22.71	4.70 x 10^5
	NaIO4 (1 mM)	200	13	22.18	5.66 x 10^5
	Initial load[c]		*7.4*	*30.5*	*7.9 x 10^3*
8	*Control*	-	*9*	*27.57*	*4.90 x 10^4*
	PK (500U)	16.6	5	29.07	1.94 x 10^4
	PK (1500U)	50	7	27.65	4.80 x 10^4
	PK (5000U)	166.6	7	27.05	7.02 x 10^4
	PK (5000U) (48h)	166.6	34	28.52	1.81 x 10^4
	Initial load[c]		*18.7*	*29.5*	*1.0 x 10^4*
9	*Control*	-	*14*	*28.22*	*2.20 x 10^4*
	α-Amylase	300	62	27.10	4.56 x 10^4
	α-Amylase	3000	20	29.93	8.49 x 10^3
	α-Amylase	6000	43	31.49	4.45 x 10^3
	Initial load[c]		*5.8*	*30.7*	*1.2 x 10^3*
10	*Control*	-	*7*	*31.48*	*7.24 x 10^2*
	α-Amylase	6000	13	31.55	6.94 x 10^2
	α-Amylase (48h)	3000	18	31.51	7.10 x 10^2
	α-Amylase (48h)	6000	6	30.62	1.25 x 10^3
Gills					
Trial	**Compound**	**mg/L[a]**	**Eff. Ext.[b](%)**	**Mean Ct value**	**RNAc/ gDT**
	Initial load[c]		*2.2*	*33.5*	*1.9 x 10^2*
7	*Control*	-	*2*	*34.10*	*1.71 x 10^1*
	NaIO4 (0.05 mM)	10	2	36.05	4.97 *x 10^1*
	NaIO4 (0.1 mM)	20	1	34.74	1.08 *x 10^2*
	NaIO4 (0.5 mM)	100	1	32.36	5.60 *x 10^2*
	NaIO4 (1 mM)	200	2	32.22	5.34 *x 10^2*
	Initial load[c]		*3.3*	*38.6*	*<LQ*
8	*Control*	-	*7*	*36.50*	*1.83 x 10^2*
	PK (500U)	16.6	7	37.52	9.11 *x 10^1*
	PK (1500U)	50	5	36.79	1.38 *x 10^2*
	PK (5000U)	166.6	5	35.31	3.92 *x 10^2*

[a]Compounds tested and their final concentrations; [b]The extraction efficiency; [c]NoV concentration following bioaccumulation (no treatment or depuration); [d]Results (expressed as Ct values, and RNA copies/g DT) for each trial are shown. PK = proteinase K. $NaIO_4$ = sodium periodate.

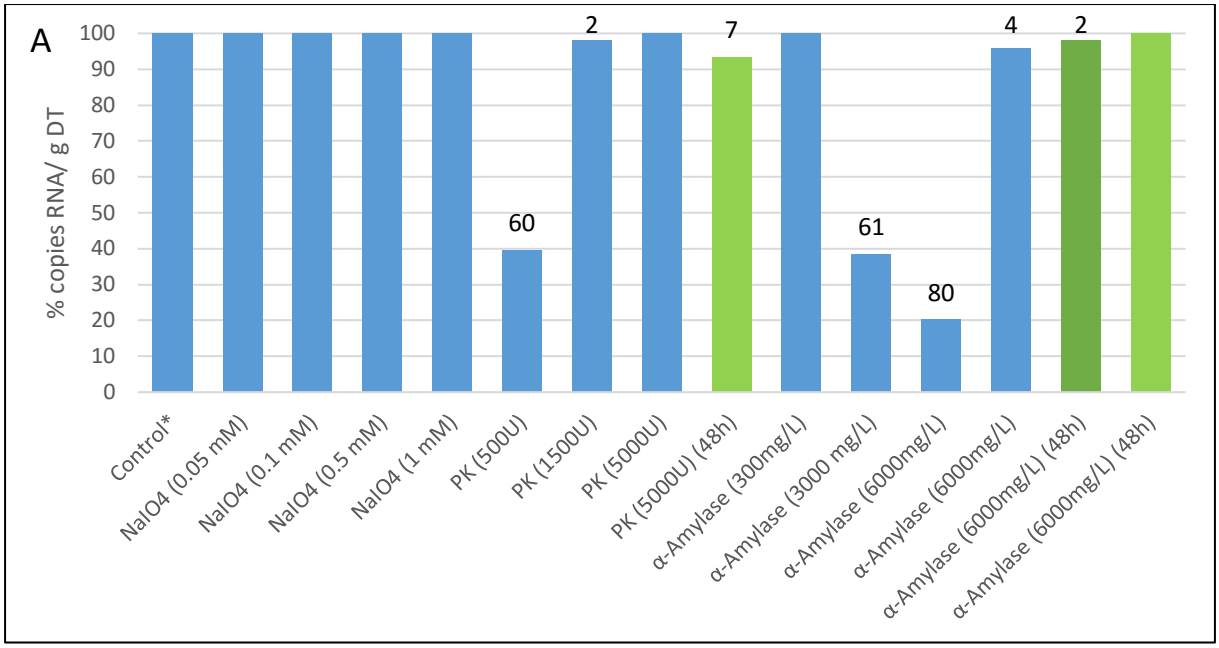

Figure 2.3: NoV GII.3 reductions in oyster DT following a 2-hour enzyme treatment step and depuration at 14°C. The bars show the amount (as a %) of NoV remaining in oysters following treatment when compared to control samples (which were not subjected to enzymatic pre-treatment) i.e. smaller bars indicate a larger reduction in NoV. Values above bars show the decrease in NoV (as a %), compared to control samples. Blue bars = depuration for 24 hours; green bars = depuration for 48 hours.

3.4 Evaluation of compound efficacy using an *in vitro* method

The efficacy of four compounds was evaluated using an *in vitro* method (Section 2.3), namely $NaIO_4$, PK, α-amylase and papain, at different concentrations (Table 2.5, Figure 2.4). The results show reductions in NoV GII.3 in the DT component in wells treated with $NaIO_4$, PK and to a lesser extent with papain. The highest concentration (100 mM) of $NaIO_4$ resulted in a decrease of NoV (RNA copies) in the DT component of about 10 fold (on average). However, the concentrations in the ST did not increase proportionately, suggesting a disruption of the capsid when high concentrations are used. Lower concentrations of $NaIO_4$ had a reduced effect.

Only the highest concentration of PK (1000 mg/L) showed a reduction in RNA copies in the DT component. Similarly, for papain only the highest concentration (100 g/L) resulted in a decrease in NoV. The *in vitro* experiments with α-amylase showed no differences in NoV concentration in the DT component following treatment (Table 2.5, Figure 2.4), confirming the results of the depuration trials.

The average concentration of virus in the corresponding ST for each treatment generally increased proportionately with the enzyme concentration, which indicates the release of NoV from the DT and its migration to the ST. This was generally the case for $NaIO_4$ (except the highest concentration), α-amylase and papain. Interestingly for PK, when the concentration of virus in the DT component decreased, levels in the ST also decreased and this was proportionate to the enzyme concentration (Figure 2.4). The observed reduction in NoV in the ST may indicate that the virus is being destroyed by the PK.

With regard to *in vitro* assays carried out with gill tissue, only $NaIO_4$ and PK were tested. While $NaIO_4$ was ineffective in reducing NoV levels, all concentrations of PK resulted in small decreases of NoV in the gill component (Table 2.5).

Table 2.5. Efficacy of four compounds in reducing NoV levels in digestive tissue (DT) (top) and gills (bottom), as determined using an *in vitro* method. Results are expressed as NoV GII.3 RNA copies/200 mg of DT or gill component, or 500 µL of supernatant (ST) along with the corresponding standard deviation (*SD*). A decrease in RNA copies in the DT or gill component when compared to control concentrations indicates that the treatment is effectively cleaving the virus/ligand from the DT.

Digestive Tissue						
Trial	Compound	mg/L[a]	DT component	*SD* of DT component	ST component	*SD* of ST component
1	*Control*	-	*4.64 x 10³*	± 2.67E+03	*6.58 x 10²*	± 5.72E+02
	$NaIO_4$ (1 mM)	200	2.27 x 10³	± 1.34E+03	1.13 x 10³	± 1.02E+03
	$NaIO_4$ (10 mM)	2000	4.11 x 10²	± 9.59E+02	2.92 x 10²	± 4.18E+02
	$NaIO_4$ (100 mM)	20000	1.99 x 10²	± 1.65E+02	1.03 x 10³	± 7.66E+02
2	*Control*	-	*1.50 x 10⁴*	± 1,50E+04	*3.90 x 10³*	± 9,24E+02
	$NaIO_4$ (10 mM)	200	3.24 x 10⁴	± 8,48E+03	7.02 x 10³	± 1,93E+03
	$NaIO_4$ (100 mM)	20000	9.76 x 10²	± 9,70E+02	7.08 x 10³	± 1,51E+03
3	*Control*	-	*6.91 x 10⁵*	± 4,98E+05	*7.45 x 10⁴*	± 1,73E+04
	$NaIO_4$ (0,05 mM)	10	3.19 x 10⁵	± 1,36E+05	4.00 x 10⁴	± 2,35E+04
	$NaIO_4$ (0,1 mM)	20	8.51 x 10⁵	± 5,12E+05	9.94 x 10⁴	± 3,46E+03
	$NaIO_4$ (0,5 mM)	100	3.82 x 10⁵	± 1,15E+05	4.11 x 10⁴	± 1,30E+04
	$NaIO_4$ (1 mM)	200	5.04 x 10⁵	± 2,08E+05	1.56 x 10⁵	± 9,18E+04
	$NaIO_4$ (10 mM)	2000	2.84 x 10⁵	± 1,75E+05	3.42 x 10⁴	± 6,63E+03
4	*Control*		*6.69 x 10³*	± 3.52E+03	*1.23 x 10³*	± 6.10E+02
	PK (0,1U)	10	5.80 x 10³	± 6.52E+03	9.79 x 10²	± 9.66E+02
5	*Control*		*2.69 x 10⁵*	± 1,68E+05	*2.07 x 10⁴*	± 8,66E+03
	PK (1,5 U)	100	5.42 x 10⁴	± 1,35E+05	1.23 x 10⁴	± 2,02E+04
	PK (7,5 U)	500	1.28 x 10⁵	± 1,93E+05	1.01 x 10⁴	± 1,24E+04
	PK (15 U)	1000	3.60 x 10⁴	± 7,00E+04	5.49 x 10³	± 1,91E+04
6	*Control*		*7.35 x 10³*	± 4,99E+03	*2.18 x 10³*	± 6,48E+02
	α-Amylase	600	1.13 x 10⁴	± 4,89E+03	3.99 x 10³	± 2,84E+03
	α-Amylase	6000	6.36 x 10³	± 2,21E+03	3.26 x 10³	± 2,90E+03
	α-Amylase	12000	1.21 x 10⁴	± 5,44E+03	3.52 x 10³	± 1,06E+03
	Control		*3.58 x 10⁴*	± 1.56E+04	*1.09 x 10³*	± 6.10E+02
	α-Amylase	20000	3.26 x 10⁴	± 2.47E+03	2.30 x 10³	± 9.19E+02
7	*Control*		*1.67 x 10⁴*	± 1.42E+04	*1.34 x 10³*	± 9.28E+02
	Papain	10000	1.01 x 10⁴	± 2.23E+03	1.47 x 10³	± 1.15E+03
	Papain	100000	4.92 x 10³	± 6.12E+03	2.58 x 10³	± 5.00E+03
Gills						
Trial	Compound	mg/L[a]	Gill component	*SD* of gill component	ST component	*SD* of ST component
1	*Control*	-	*3.69 x 10²*	± 2,91E+02	*1.64 x 10²*	± 3,50E+02
	$NaIO_4$ (0.05 mM)	10	5.59 x 10²	± 6,10E+02	3.08 x 10¹	± 2,18E+01
	$NaIO_4$ (0.1 mM)	20	5.60 x 10²	± 3,69E+02	1.52 x 10²	± 1,66E+02
	$NaIO_4$ (0.5 mM)	100	7.74 x 10²	± 9,20E+02	4.37 x 10¹	± 1,30E+02
	$NaIO_4$ (1 mM)	200	3.97 x 10²	± 2,53E+02	1.30 x 10²	± 1,18E+02
	$NaIO_4$ (10 mM)	2000	3.50 x 10²	± 2,06E+02	9.18 x 10¹	± 7,53E+01
2	*Control*	-	*2.28 x 10²*	± 2,73E+02	*<LQ*	-
	PK (1.5 U)	10	<LQ	-	<LQ	-
	PK (7.5 U)	50	1.41 x 10²	± 4,22E+01	<LQ	-
	PK (15 U)	1000	1.10 x 10²	± 1,09E+02	<LQ	-

[a]Compounds tested and their final concentrations. LQ = limit of quantification (50 RNAc/g of DT). PK = proteinase K. $NaIO_4$ = sodium periodate.

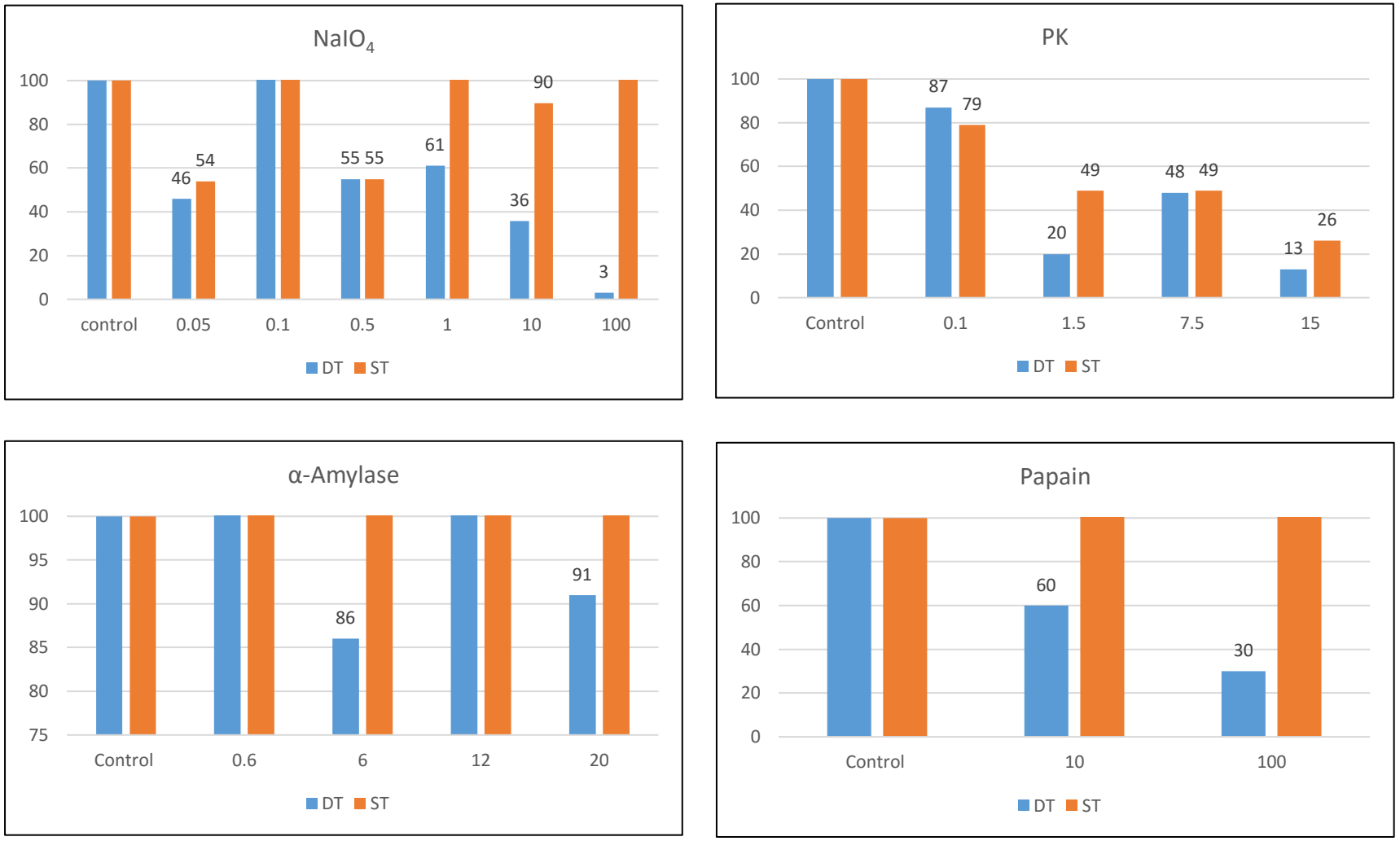

Figure 2.4: Comparison of NoV GII.3 levels (detected by an *in vitro* assay) in the digestive tissue (DT) (blue bars) and supernatant (ST) components (orange bars) of oysters treated with various compounds. Levels are expressed as a % of that detected in control wells in which no compounds were applied. Compound concentrations are expressed in mM for $NaIO_4$, in units for PK (proteinase K), and mg/L for α-amylase and papain.

4 Discussion

Two different types of experiments were undertaken to identify the best candidate compounds: (1) live oysters contaminated with NoV were held in tanks, a selection of compounds were added at different concentrations using a range of conditions, and depuration was then undertaken; and (2) *in vitro* assays were conducted to evaluate the potential for the compounds to reduce NoV levels in the digestive and gill tissues.

The study began with the selection of compounds that can potentially disrupt NoV ligands in oyster tissues, that are safe for consumers and environmentally friendly. Norwalk virus (GI.1) binds to the DT of *C. gigas* via an A-type HBGA (Maalouf et al., 2010b). As in humans, NoV GII recognises a slightly different ligand and displays a different tissue distribution than GI strains. Higher concentrations of GII are detected in gill and mantle tissues, where binding involves SA, whereas in the DT the interaction involves both SA and an A-type HBGA (Maalouf et al., 2010b; Zakhour et al., 2010). Based on this knowledge, $NaIO_4$ was selected for further investigation because it cleaves sialylated carbohydrate motifs. PK was selected because of its efficiency in disrupting viral particles as part of the extraction step in different analytical methods. Enzymes such as papain and α-amylase were selected because of their activity against HBGAs, and also the ability to readily source these compounds for trials.

Secondly, consideration was given to the strains of NoV to use for the experiments. NoV GII.3 and GI.1 were chosen because they are efficiently bioaccumulated by oysters (Maalouf et al., 2011; Yu et al., 2015). All experiments were thus performed with two stools that have been previously characterised and are known to exclusively contain GII.3 and GI.1 NoV (Drouaz et al., 2015; Maalouf et al., 2011; Maalouf et al., 2010b). This approach eliminated variability that could be due to the presence of different NoV strains and varying bioaccumulation efficiencies in oysters.

Quantification of the viruses in oysters was conducted using real-time RT-PCR, following the ISO/TS standard method. Standard precautions were adopted to ensure accuracy, including multiple extractions in different series, incorporation of quantified nucleic acid standards and the use of inhibition controls. However, as with many microbiological quantification techniques, comparing levels within a log can be challenging due to the variability of the method. Thus, small changes in viral levels could be 'masked' by method variability, as suggested by some results observed in this study (particularly for gill samples which had low levels of NoV). During a recent field study, for which triplicate extractions and triplicate amplifications of each extract were performed, high variability was observed for oysters with lower levels of contamination (Le Mennec et al., 2016). To avoid such variability and be within the working range of the method, oysters in this study were contaminated at quite high levels, which may not be representative of usual contamination levels.

One of the main challenges in using enzymes for treating NoV contaminated oysters is the difficulty associated with optimising conditions to maximise enzyme activity. Thus, some depuration trials were conducted at higher temperatures (20°C and 27°C) than those routinely used in the UK; this raises the possibility that observed NoV reductions may be partially due to the high temperature and potential increases in oyster filtration rates. However, the control oysters, to which no enzyme treatment was applied, showed no noticeable NoV depuration, even at 27°C. This confirms that the effect (i.e. enhanced depuration) observed for some compounds, particularly PK, trypsin and papain, was not related to increases in physiological activity of the oysters alone. PK also showed an effect at the lower temperature of 14°C (trypsin and papain not tested at lower temperatures). $NaIO_4$ improved the NoV depuration rates at 20°C or 27°C, but there was no noticeable effect at 14°C. For $NaIO_4$, the increased physiological activity of the oysters at higher temperatures may have contributed to the higher depuration rates observed (Bougrier et al., 1995), because there is no evidence to suggest that the activity of $NaIO_4$ increases at higher temperatures. It should also be noted, that temperature is

not the only parameter influencing the activity of the compounds used in this study, other factors such as the pH of the seawater may also have an impact and could be investigated in future trials.

The conditions (e.g. temperature) used during treatment and depuration are important to consider, as good filtration activity needs to be maintained to ensure that the compounds reach the target tissue and exert their effect on the HBGA-virus bond. Most compounds tested showed no effect on oyster filtration activity, however we did note that the lipase and amylase treatments formed foam upon aeration resulting in slightly foggy seawater. In some trials, $NaIO_4$ impacted the oysters filtration behaviour, when oysters came in contact with $NaIO_4$ they closed their valves and ceased filtering. This behaviour was observed for all concentrations used with $NaIO_4$, but it was more striking for the highest level. This may explain why NoV reduction was highest in DT using a low $NaIO_4$ concentration, rather than higher concentrations. We did not observe a significant reduction in NoV in the gills of oysters treated with $NaIO_4$, the reduction in oyster filtering may have impaired any potential reductions making it difficult to evaluate the effect of the $NaIO_4$. This finding highlights the need to find a balance between the optimal conditions for the compound activity, such as concentration, solubility in seawater, temperature and the physiological conditions of the oysters to maximise their filtration activity. These factors, in addition to the physiological condition of the oysters at the outset, may also contribute to variability in NoV levels.

To further evaluate the efficacy of the selected compounds an *in vitro* method was developed that demonstrated that PK and papain can reduce NoV GII.3 levels in DT. This corroborated results from the depuration trials, which showed that PK, papain and trypsin were effective in enhancing depuration (note, trypsin was not trialled using the *in vitro* method). The *in vitro* test also demonstrated that $NaIO_4$ can reduce GII.3 levels in DT (similar to depuration trials at elevated temperatures), however very high concentrations of $NaIO_4$ were needed for significant elution of GII.3, exceeding $NaIO_4$ levels needed to destroy SA ligands (Maalouf et al., 2010b). This observation may suggest that after 24 hours bioaccumulation, the NoV particles are not just binding to SA, but may also be entrapped in the tissue. Depuration trials for $NaIO_4$ gave mixed results, with no enhanced reductions at 14°C and negative impacts on oyster filtration. Thus, despite the advantages of $NaIO_4$ being a chemical compound of a non-enzymatic nature, which is more stable in seawater and able to disrupt/destroy SA, this compound is not a good candidate. PK and papain (plant proteases) showed the most promise in both the depuration trials and *in vitro* assays. This raises the question of whether plant proteases are more resistant than animal proteases to seawater conditions and thus potentially more adequate for use in depuration processes.

The impact of longer contact times with some compounds (including PK) was evaluated in this study, with the 'dipping' period increased from one to two hours. The depuration period was also lengthened, from 24 hours used in initial experiments, to a 48-hour period (more similar to the 42 h period routinely practiced in the UK). However, the increments in dipping and depuration times did not further enhance the reduction of NoV.

5 Conclusions and Recommendations

This study aimed to identify compounds that enhance NoV depuration and provides promising initial results to support future in-depth investigations. Firstly, from a technical perspective, the development of an *in vitro* method will be useful for further studies. Secondly, the *in vitro* method and depuration trials identified two plant proteases, PK and papain, that show promise in reducing NoV in oysters. Depuration trials demonstrated that trypsin may also be effective. While the initial studies are encouraging, further research is required to confirm and optimise the effect of these compounds, thus the following specific recommendations are offered:

1. The preliminary experiments were conducted on oysters contaminated with high levels of NoV – this enabled the reduction in NoV to be accurately quantified in most trials. Further experiments are required on oysters that contain lower levels of NoV, such as those that are naturally contaminated, to confirm the effect of these compounds in all oyster tissues. Quantification of low levels of NoV in DT and other tissues using the existing real time RT-PCR method is subject to considerable variation, thus it is likely that future studies would need to use a more precise method. Recent advances have been made on the development of a digital PCR (dPCR) method for the detection of NoV in oysters (Polo et al., 2016). This method precludes the need for standard curves and is less prone to inhibition, and thus may be less variable that the standard ISO/TS real time PCR based method. Additionally, the dPCR method has been used for naturally contaminated oysters, and it may be suitable for future studies on the impact of various compounds (Polo et al., 2016).

2. Further studies that seek to optimise the physiological activity of oysters and the enzymatic activity of PK, papain and trypsin should be conducted, including optimisation of conditions such as seawater temperature, pH, enzyme concentration, and dipping and depuration periods.

3. Consideration could be given to conducting experiments which involve the immersion of contaminated oyster batches for short periods in warm seawater, at temperatures at which the enzymes will be more efficient (generally >30°C). Higher temperatures than those trialled to date (27°C) could be tested, but for very short times to avoid spawning or increases in the growth of pathogenic bacteria such as Vibrio.

4. A key issue to consider when applying treatments such as enzymes to oysters, is the need to ensure that adequate amounts of enzyme reach the sites of interest inside the oyster. Microencapsulation (Part 1, Section 6.2.3) has been used for the delivery of vaccines and probiotic bacteria in the aquaculture sector and holds potential for ensuring active enzymes reach the HBGA-NoV complexes in oysters. Future studies could be conducted to explore the feasibility of microencapsulation of the enzymes, and to evaluate if this facilitates further NoV reductions in oysters.

Lastly, while enzymatic pre-treatment holds potential for enhancing NoV reduction through the depuration process, it is clear that NoV can persist for long periods in oysters due to the specific interactions between HBGAs and NoV. Therefore, it is considered that the major focus should be placed on improvements in water quality to avoid NoV and HAV contamination of shellfish at source.

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Appendix One: Evaluation of key publications considered in the literature review

An evaluation of the most relevant papers considered in the literature review was undertaken (Tables A1 and A2). The scientific findings of each paper are discussed in the literature review section of this report (Part 1). The methodology (Section 2) provides details on the selection process for publications included in the evaluation and the approach used to evaluate the papers. Briefly, the selected papers were critiqued against a series of pre-determined questions. The questions used to evaluate the papers were:

Question 1: Were appropriate analytical test methodologies used for NoV (i.e. the ISO standard method or equivalent)?
Question 2: Were depuration parameters noted in the study (e.g. temperature, salinity, time, disinfection approach, flow rate, loading density)?
Question 3: Did the study design, data and statistical treatment support the conclusions?

For NoV uptake and depuration studies:
Question 4: Were oysters maintained in appropriate conditions to ensure they were alive and functioning optimally?
Question 5: Was the number of sampling occasions and/or oyster samples analysed sufficient to support conclusions regarding relative elimination efficiency for NoV?

For reports on illness outbreaks related to NoV in oysters:
Question 4: Does the epidemiology evidence presented strongly implicate oysters as the vector (i.e. were the epidemiology investigations analytical and have statistics presented)?
Question 5: Were oysters tested for the presence of NoV?

For each of the papers considered, the questions above were assessed and a score of 0 (no), 1 (acceptable/generally) or 2 (yes) was allocated for each question. A total score was calculated for each paper, thus high scoring papers are suggestive of robust results and conclusions (a maximum score of 10 is possible). Tables A1 and A2 provide the results for the evaluation.

Table A1 Summary of evaluation undertaken for key norovirus depuration publications considered in the literature review

	Q1: Appropriate methods used?	**Q2:** Depuration parameters noted?	**Q3:** Study design appropriate to support conclusions?	**Q4:** Oysters maintained appropriately?	**Q5:** Sampling numbers sufficient?	TOTAL SCORE
McLeod et al. (2009a)	2 (real time PCR)	2	2	2	1 (sampling could have been extended beyond 24 hours)	**9**
Schwab et al. (1998)	1 (PCR, semi-quan)	2	2	2	2	**9**
Drouaz et al. (2015)	2 (real time PCR)	1 (type of system not noted)	2	2	2	**9**
Ueki et al. (2007)	2 (real time PCR)	2	2	2	2	**10**
Neish et al. (2013)	2 (real time PCR)	2	1 (A statistically significant reduction in NoV noted, but the small decrease may have been biologically insignificant)	2	2	**9**
Choi and Kingsley (2016)	2 (real time PCR)	2	2	2	2	**10**
Drouaz et al. (2015)	2 (real time PCR)	2	2	2	2	**10**

Table A2 Summary of evaluation undertaken for key NoV outbreak publications considered in the literature review

	Q1: Appropriate methods used?	**Q2:** Depuration parameters noted?	**Q3:** Study design appropriate to support conclusions?	**Q4**: Is epidemiological evidence strong?	**Q5:** Were oysters tested for NoV?	**TOTAL SCORE**
Rajko-Nenow et al. (2014)	2	0	2	1 (not specified)	2	**7**
Fitzgerald et al. (2014)	2	0	2	1 (descriptive)	2	**7**
Dore et al. (2010)	2	0	2	1 (not specified)	2	**7**
Smith et al. (2012)	2	0	2	2 (analytical)	2	**8**
Le Guyader et al. (2010)	2	0	2	2 (analytical)	2	**8**
Lowther et al. (2010)	2	0	2	1 (not specified)	2	**7**
Huppatz et al. (2008)	1 (not specified)	1 (time and disinfection noted)	2	2 (analytical)	1 (different batch tested)	**7**
Le Guyader et al. (2008)	2	0	2	2	2	**8**
Gallimore et al. (2005)	NA	1 (time, temp noted)	2	1 (descriptive)	0	**4**
Le Guyader et al. (2006b)	2	0	2	2	2	**8**
Ang (1998)	NA	0	2	2	0	**4**
Stafford et al. (1997)	1 (not specified)	0	2	2	1 (tested, not detected)	**6**
Heller et al. (1986)	NA	1 (time, UV noted)	2	2	0	**5**
Gill et al. (1983)	NA	2	2	2	0	**6**
Grohmann et al. (1981)	NA	1 (time noted)	2	2	0	**5**

Appendix Two: International and UK depuration surveys

Part 1: UK Depuration Systems Questionnaire

Seafood Safety Assessment Ltd
'Hillcrest', Kilmore, Isle of Skye, IV44 8RG
E-mail: seafoodsafetyassessment@gmail.com
Tel: 01471 844 725

Seafood Safety Assessment Ltd (SSA Ltd) has been commissioned by the UK Food Standards Agency (FSA) to review the effectiveness of depuration in removing norovirus from oysters and to investigate alternative approaches to virus removal.

One element of this study is to identify current depuration practice in the UK, including whether the system is operator designed/constructed or purchased 'off the shelf' from one of the established manufacturers and actual operational parameters of temperature, UV power, oxygen levels, use of ozone, length of depuration cycle, etc. The Questionnaire (attached) also aims to gather industry observations about 'real world' problems and issues concerning the practical operation of these systems.

The output from this research into current depuration systems aims to be a comprehensive summary of the typical values and range of current operational criteria, plus a synopsis of industry concerns associated with the process.

Completion of this Questionnaire by you, a licensed depuration operator, will contribute significantly to the accuracy and all-inclusive nature of the output. In addition, the responses will enable researchers to 'replicate' commercial reality when testing potential alternative approaches to virus depuration.

SSA Ltd is grateful for your participation in this research, which will assist in efforts to improve the public health status of oysters harvested in the UK. The survey of industry practice is supported and endorsed by the main representative trade organisations, the Shellfish Association of Great Britain (SAGB) and the Association of Scottish Shellfish Growers (ASSG).

Completed Questionnaires should be returned to the address above by the end of March 2015 at the latest. Please note that individual company responses will remain confidential and will not be published – only a summary of UK-wide results will be incorporated into the project.

Questionnaire on Oyster Depuration Practices in the UK

Name:	
Position/role:	
Company Name:	
Address:	
E-mail:	
Phone:	
Mobile:	

Please place a cross in the boxes and/or complete answers in the space provided.

1. In which year was your depuration system commissioned?_____

2. Is your depuration system self-designed/constructed?

Yes ☐ *If Yes, Go to Q.4*
No ☐

3. Is your depuration system a standard Seafish 'off the shelf' design, from a third party manufacturer?

Yes ☐ *If Yes, please identify the manufacturer:* _____
No ☐

4. Which species of oysters are depurated at your location?

Crassostrea gigas ☐
Ostrea edulis ☐
Other ☐ *Please specify:* _____

5. How are the shellfish loaded into the depuration system?

Loose on trays ☐
Bags/sacks ☐
Small mesh boxes ☐
Other ☐ *Please specify:* _____

6. What is the stocking density/container? _____ Kg per Tray/Bag/Box

7. What is the maximum amount of oysters (in Kilograms) that can be purified in the system in a single cycle? _____Kg

8. What volume of water is contained in each tank (_____m3) and the total system (_____m3) when fully loaded with oysters?

9. Typically, how long (hours) is each depuration cycle?

- In summer:_____Hrs.
- In winter: _____ Hrs.

10. What is the flow rate through the system during the cycle period?

- In summer:_____Litres/sec
- In winter:_____Litres/sec

11. At what water temperature(s) do you operate the system?

- Max:_____
- Min:_____

12. Do these vary between summer and winter operation?

Yes ☐
No ☐

If Yes, what are the seasonal temperatures:

- Summer Max:_____ Min:_____
- Winter Max:_____ Min:_____

13. At what dissolved oxygen level(s) in the tank water do you aim to operate at (start/finish of cycle)?

- Start of cycle: _____mg/L
- Finish of cycle:_____ mg/L.

14. Is the water supply for the depuration process locally sourced seawater, tanker supplied or artificial seawater?

Local supply ☐
Tanker supplied ☐
Artificial ☐

15. What is the typical salinity of the seawater used? _____ ‰

16. Do you test the water supply (before UV treatment) for:

- Microbiological contamination?

Yes ☐
No ☐

- Turbidity?

Yes ☐
No ☐

If Yes, what threshold level(s) would cause the depuration process to be halted?

- Microbiological:_____
- Turbidity:_____

17. Do you use in-line filters on inlet water pipework?

Yes ☐
No ☐

If Yes, are the filters:

Cartridge ☐
Sand ☐

18. Do you re-use seawater after completion of a cycle?

Yes ☐
No ☐

If Yes, how many times is the water typically reused? _____

19. What power of UV lamps is typically used? _____

20. What dose does this result in:

- New UV lamp? _____mJ/cm2
- At 80% efficiency? _____mJ/cm^2

21. Do you use ozone treatment?

Regularly ☐ *If Yes, at what concentration?* _____
Occasionally ☐ *If Yes, at what concentration?* _____
Never ☐

22. What problems/difficulties/issues have you experienced with the practical operation of depuration facilities? _____

23. Do you believe that depuration is potentially an effective process to remove viruses from oysters and if so, what criteria (temperature, cycle time, oxygen levels, etc.) would be required to effect significant reduction? _____

24. Have you participated in any viral reduction studies/projects using your depuration system?

Yes ☐
No ☐

If Yes, can you supply a reference/publication for the research: _____

25. Would you be willing to discuss the issues surrounding viral reduction through depuration and/or alternative approaches with a representative of SSA Ltd?

Yes ☐
No ☐

If Yes, please indicate the best day and time for an initial telephone contact: _____

SSA Ltd appreciates the time and effort taken to complete the Questionnaire, which we hope will prove to be a significant contribution to improving the quality of oysters placed on the market.

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Part 2: International Depuration Questionnaire

Seafood Safety Assessment Ltd
'Hillcrest', Kilmore, Isle of Skye, IV44 8RG
E-mail: seafoodsafetyassessment@gmail.com
Tel: 01471 844 725

Seafood Safety Assessment Ltd (SSA Ltd) has been commissioned by the UK Food Standards Agency (FSA) to review the effectiveness of depuration in removing norovirus from oysters and to investigate alternative approaches to virus removal.

One element of this study is to identify current depuration practices in the UK; in addition the project aims to include an international comparison of operational parameters. To this end SSA Ltd would be grateful if you could take the time to complete (as far as relevant) this questionnaire on depuration operations as practiced by the oyster industry (producers and processors) in your country. If you are interested in further background information on the project, please visit:
http://www.food.gov.uk/science/research/foodborneillness/p01programme/fs101068

Questionnaire on Depuration Systems

Name:……
Position/Role:……..
Organisation:……
Address:……..
Contact Details: E-mail ……..; Tel: ……

1. What proportion of oysters placed on the market in your country would you estimate are depurated (defined as: 'Purification carried out under controlled conditions')?
   All
   75%
   50%
   25%
   None

   Comments:

2. Are the depuration systems used by industry generally 'self-constructed' or "off the shelf" purchases from third party manufacturers using recognised technical standards? If the latter, which manufacturers, whose standards?
   Off the shelf
   Self constructed

   Comments:

3. What is the scale of depuration operations, i.e. what amount of oysters (Kilograms) can be purified in a typical system in a single cycle?

   - Average:……..Kg/cycle
   - Range:……..Kg/cycle

4. Which species of oyster are generally depurated, e.g. Crassostrea gigas, Ostrea edulis, etc.? ……
   ……

5. For each oyster species depurated, could you specify typical parameters as listed below:

   - How long is the depuration cycle for each species? …….
   ……
   - At what temperatures are the systems operated? ……..
   ……
   - At what salinity are the systems operated? ……..
   ……
   - Is ozone used (Frequently? Occasionally?) and at what concentration(s)?
   ……..
   ……

Species	Depuration period (hours)	Seawater temperature	Salinity	Ozone used	Ozone concentration
				Yes, no, sometimes	

6. Is there anything unique or different about the design or operation of depuration units in your country when compared with other international operators?

7. Have there been any problems/difficulties/issues experienced by operators with the practical operation of depuration facilities?
   ……
   ……
   ……

8. Are you aware of any norovirus outbreaks associated with depurated oysters from your country? Yes/No

   If 'Yes', please provide details of any associated publications/reports.

9. Are you aware of any research/experimental work/pilot projects targeting removal of Norovirus from oysters through depuration or other methods(published or unpublished)? Yes/No

   If Yes, please provide details if possible ……..
   ……

10. What are the references for any published studies/test results for work investigating removal of Norovirus? ……
    ……

SSA Ltd appreciates the time and effort taken to complete the questionnaire.

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Appendix Three: Summary of compounds that may act as disruptors of NoV ligands in oyster tissues

Origin	Compound (synonymous)	Source	Compound family	Cleavage site	pH optimum	Observations
Animal proteases	Lipase	Porcine/Bovine pancreas, *Candida rugosa*	Esterase	Esters in aqueous solutions, hydrolysis of triacylglycerols	------	Ca2+ necessary for activity Also from plant, bacterial and fungi origin
	Trypsine	Porcine/Bovine pancreas	Serine Endoprotease	Peptides on the C-terminal side of Lys and Arg amino acid residues	7.0-9.0	Ca2+ retard the autolysis ability and maintain the stability in solution
	α-chymotrypsine	Bovine pancreas	Serine Endoprotease	Peptide bonds on the C-terminal side of Tyr, Trp, Phe, Leu	------	Ca2+ activates and stabilizes the enzyme
	Pronase XIV (Pronase E)	*Streptomyces grisseus*	Endo/exo-proteases	Non specific protease	7.0-8.0	Ca2+ is recommended for protection from autolysis. Much more effective in digestion of casein than trypsine, chymotrypsine and other proteases.
	O-sialo glycoprotease	*Pasteurella haemolytica*	Endo glycosidase	O-glycosylated proteins on Ser and thr residues, removing sialyl groups	7.4	
Plant proteases	Proteinase K	*Tritirachum album*	Serine Endoprotease	Peptide bonds adjacents to the carboxyl group of aliphatic and aromatic amino acids	7.5-12	Ca2+ necessary for activation (1-5mM Ca2+) Maximum activity at 37ºC; 80% loss of activity at 20-60ºC
	Papain	Papaya latex *Carica papaya*	Cysteine Endoprotease	Peptide bonds of basic amino acids, esters and amides, especially at bonds involving Arg, Lys, Glu, His, Gly and Tyr.	6.0-7.0	Much more effective than pancreatic proteases. Upon prolonged incubation further bonds are split. The addition of L-cystein (0.5% w/v; 5mM) is essential for enzyme activity.
	Ficin	Fig tree latex	Thiol protease	Carboxyl side of Gly, Ser, Thr, Met, Lys, Arg, Tyr, Ala, Asn, Val	------	
	Bromelain	Pineapple stem	Cysteine Endoprotease	Broad protein specificity	------	
Glycosidases	α-Amylase	Porcine/Human pancreas	Endo glycosidase	α(1,4)-D-glucosidic linkages in polysaccharides of ≥3 of α(1,4) linked D-glucose units	7.0	Ca^{2+} necessary for stability; Chlorine ions necessary for stability; Also from human saliva and Bacterial origin
	B-Galactosidase (Lactase)	*Bacteroides fragilis,* expressed in *E. coli*	Endo glycosidase	β-glycosidic bond formed between a galactose and its organic moiety	6.0-8.0	Mg^{2+} and Na^{2+} are activators. The optimal concentration of Mg^{2+} can range from 0,1 to 10 mM, depending upon the sodium concentration
	O-Glycosidase	*Streptococcus pneumoniae*	Endo glycosidase	O-Glycans. N-acetylgalactosamine glycosidic linkage	6.0-8.0	

Origin	Compound (synonymous)	Source	Compound family	Cleavage site	pH optimum	Observations
	Neuraminidase (Sialidase)	*Vibrio cholerae*	Exo glycosidase	Preferentially α(2→3), but also α(2→6) and α(2→8) linkages between neuraminic acid and galactose	5.0	Ca^{2+} necessary for activity
	α-L Fucosidase	Bovine kidney	Exo glycosidase	α(1→ (2,3,4,6)) linked fucose from N- and O linked glycans.	5.5-5.8	It cleaves α-1→6 linked fucose on the trimannosyl core of N-linked glycans more efficiently than other α-fucose linkages.
	$NaIO_4$		Sodium salt of periodic acid	Sialic acids and carbon-carbon bonds of a wide range of carbohydrates	------	more effective in dark conditions

Appendix Four: Reproducibility of the *in vitro* method

Table A4 NoV levels in digestive tract (DT) and supernatant (ST) components of samples treated with three concentrations of sodium periodate ($NaIO_4$) in 24-well cell culture plates and Eppendorf tubes. Results are expressed as NoV GII.3 RNA copies/200 mg of DT component, or 500 µL of ST along with the corresponding standard deviation (*SD*).

Assay format	Compound	mg/L[a]	DT component	*SD* of DT component	ST component	*SD* of ST component
24-well plate	*Control*	-	4.36×10^3	$\pm 1.61 \times 10^3$	4.42×10^2	$\pm 2.34 \times 10^2$
	$NaIO_4$ (1 mM)	200	1.32×10^3	$\pm 2.27 \times 10^2$	4.09×10^2	$\pm 2.82 \times 10^2$
	$NaIO_4$ (10 mM)	2000	1.47×10^2	$\pm 5.26 \times 10^2$	7.11×10^2	$\pm 1.54 \times 10^2$
	$NaIO_4$ (100 mM)	20000	6.55×10^1	$\pm 4.67 \times 10^1$	1.10×10^3	$\pm 1.36 \times 10^2$
Eppendorf	*Control*	-	2.49×10^3	± 6.75	4.66×10^2	$\pm 1.55 \times 10^2$
	$NaIO_4$ (1 mM)	200	2.15×10^3	$\pm 7.17 \times 10^2$	3.99×10^2	$\pm 1.22 \times 10^2$
	$NaIO_4$ (10 mM)	2000	2.02×10^3	$\pm 1.91 \times 10^2$	1.18×10^3	$\pm 1.01 \times 10^2$
	$NaIO_4$ (100 mM)	20000	9.51×10^1	$\pm 5.07 \times 10^1$	4.90×10^2	$\pm 1.32 \times 10^2$

For more information please contact:

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The Onshore Training Team

Here to give the UK seafood sector
the support it needs to thrive.

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