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Risk assessment of the use of alternative animal and plant raw material resources in aquaculture feeds

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Abstract

A wide range of raw materials are now used routinely in aquaculture feeds throughout the world, primarily to supply protein and energy in the form of lipid from edible oils. Protein meals and oils used can generally be divided into those of plant or animal origin and many have considerable potential to supply the required dietary nutrients required by aquaculture species. However, the use of any raw material introduces a suite of risks that need to be considered to enable the production of safe, sustainable and functional feeds to underpin this sector. A lack of understanding of some of those risks can result in failure of dietary specifications being met and/or negative nutritional elements being introduced (e.g. antinutritional factors). Importantly, it is this feed that when fed to food-producing animals is such an important element of food safety, and as such any undesirable aspects relating to feed production can also have a negative impact on the rest of the food chain. However, there is some disparity internationally among raw materials that are used and the perceptions surrounding the risk of their use. It is the scientific assessment of these risks that is the basis of this review.

Key words: antinutritional, BSE, contaminant, diets, fishmeal, GMO, nutrition, pathogen, risk.

Introduction

Aquaculture now produces most the world's seafood and recently became a larger contributor to the human food chain than beef production (Larsen & Roney 2013). Like all intensive animal production industries, aquaculture is heavily reliant on feed inputs to sustain its production. Traditionally, there has been much reliance on the use of wild-caught fishery products, like fishmeal and fish oil, in feeds for aquaculture species and because of this, some sectors of aquaculture have been perceived as a net fish user rather than producer (Naylor *et al*. 2009). However, in addition to alleviating concerns about the reliability of aquaculture as a food provider, and the long-term sustainability of aquaculture as an industry, the use of alternative raw materials to fishmeals and oils also empowers the formulator with additional options. Some analysts suggest that the trophic level implications through the use of these raw materials in modern feeds now means that farmed fish occupy comparatively lower trophic positions, and therefore consume less resources, than equivalent wild caught species (Tacon *et al*. 2010). Using other raw material options can also introduce the potential to improve the technical qualities of feeds and the capacity to include certain nutrients and bioactive products, thereby further increasing the value of resultant compound diets in which the ingredients are included.

Alternative protein meals and oils can generally be divided into those of plant or animal origin (although there are also now some bacterial and fungal products emerging) and many have considerable potential to supply the required dietary nutrients for aquaculture species (Bureau *et al*. 1999; Gatlin *et al*. 2007; Hardy 2010). The optimization of the use of these resources in aquaculture diets depends on a detailed understanding of the chemical composition of these products, the consequences of feeding these products and their influence on each specific species being fed (Glencross *et al*. 2007a). However, like the use of any raw materials, the use of alternative proteins and oils to those from fishery products also introduces a suite of risks that needs to be considered to enable the production of safe, sustainable and functional feeds. However, there is some disparity internationally among the raw materials that are used and the associated perceptions surrounding the risk with their use.

Some of this international disparity can be linked to the incidence of food scandals that have historically arisen because of contamination of human food either via the feed or other points in the production chain (Lloyd *et al.* 2006; Kher *et al.* 2013). Clear examples of this include the Belgian dioxin scandal in 1999, the United Kingdom (UK) mad cow scandal in 2001 and the adulteration of wheat with melamine in China. Each of these cases has shown that contaminants of either a chemical or zoonotic agent can be transferred to consumers via the feed. It was clear from incidents such as these, that feed production can potentially have an enormous negative impact on the rest of the food supply chain. Therefore, the feed provided to production animals that are consumed by humans, has become a critically important control point for overall food safety. Consequently, regulations have evolved in different regions of the world that set certain standards to regulate what raw materials are permitted in feeds for certain species. In additional to these statutory regulations, some regions/markets have also instigated 'voluntary' regulation of the use of some raw materials, based on risks to market perceptions and ideologies (Skogstad 2011; Spök *et al.* 2004). It is the assessment of these risks that is the basis of this review.

The perfect raw material?

The historical pretext to the use of fishery meals and oils was a logical one. Both raw materials are close to the 'perfect' raw material for formulating feeds, especially so for carnivorous aquaculture species, because of their high nutrient density and suitable balance of amino and fatty acids in each case. However, in assessing the potential of alternative raw materials, it is a fallacy that there needs to be search for a single ideal replacement, as this simply transfers risk from one raw material to another. A more appropriate strategy is to enable the use of a broad suite of raw materials that enables formulators' substantial flexibility to adapt to changes in supply, price and quality risks as they arise (Glencross *et al.* 2007a; Turchini *et al.* 2019). This is only achieved by developing an improved understanding of a broad range of raw materials, understanding their limitations and then applying the knowledge of those constraints against the specific nutrient demands of each of the species when diets are formulated.

Among raw materials, there has been considerable research on the use of the plant protein resources in the diets of aquaculture species (Gatlin *et al.* 2007). Soybean products are the most widely produced and used plant protein source in aquaculture diet formulations, and they have been applied with considerable success in diets for a wide range of species (Refstie *et al.* 1998, 2000; Glencross *et al.* 2004a). However, there are a range of other plant protein concentrates produced from corn, faba beans, lupins, peas and rapeseed that have value as potential aquaculture feed ingredients (Booth *et al.* 2001; Glencross *et al.* 2004b,c; Gatlin *et al.* 2007).

However, the use of plant protein resources in fish diets can also introduce a suite of problems. Not only does the use of high-levels of plant proteins increase the potential for inducing nutritional specification issues, like essential amino acid limitations, but most plant protein resources also contain a variety of biologically active antinutritional factors (ANF). The influence of these ANF on fish can be considerable and varied (Krogdahl *et al.* 2010). More recently, concerns have been raised over the use of some raw materials based on their genetic modification status, with legislation enacted in some parts of the world to limit the use of those raw materials produced from transgenic or genetically modified organisms (GMO; Spök *et al.* 2004; Skogstad 2011; Aleksejeva 2014).

Rendered animal meals, also called land-animal proteins (LAPs), are another protein resource stream that have been widely used in aquaculture diet formulations, with considerable success (Bureau *et al.* 1999; Williams *et al.* 2003a,b). However, in some regions (e.g. Europe), there has been limited use of these protein resources due to a range of legislative, policy and perception issues based on the perceived risks to human health arising principally around the concerns for introduction of transmissible spongiform encephalitis (TSE; Woodgate & Van Der Veen 2004). However, the basis for these concerns has never been adequately substantiated in any aquaculture species and there is still widespread disparity in the use of these resources throughout most of the world. Additionally, there is substantial production of animal-derived oils from some sectors and these too have potential as a feed resource, but similarly there is disparity in the use of these resources throughout the world as well (Turchini *et al.* 2009; Emery *et al.* 2014; Salini *et al.* 2015).

Moving beyond fishmeal – to jump or waiting to be pushed?

In the 21st century, the data available to underpin the potential to replace fishmeal and fish oils in aquaculture feeds is considerable by any regard. While it can be argued that the species-specific data can be patchy, across the multitude of species produced in aquaculture a comprehensive data assembly is clearly available

(Gatlin *et al*. 2007; Hardy 2010). There is also evidence that there is considerable cross-species utility of many of the data sets, though others contest this issue, and this remains an area to be further validated (Refstie *et al*. 1999, 2000; Glencross *et al*. 2004a; Glencross 2011). Given these paradigms, the question arises – why has not the industry moved to higher levels of replacement of fishery products in aquaculture feeds?

However, the issues and options associated with the replacement of either fishmeals or fish oils are far from as simplistic as they might initially appear. To be considered into the equation are issues of a commercial context such as those of price, supply and utility (a combination of biological value and palatability). These issues clearly have a broader range of drivers than many of the relatively simplistic biological drivers that underpin much of the academic research available in the public domain (Glencross *et al*. 2007a; Hardy 2010). To facilitate the adoption of alternative raw materials, the question needs to be asked, why are alternative adoptions not being made to their full potential? Beyond commercial issues of price, much of this has to do with the level of consolidated confidence in the use of any specific raw material and around the boundaries of the use of these raw materials. It is typical for many formulators to put confidence 'constraints' around the use of certain raw materials and these 'constraints' are largely placed due to confidence issues in a range of issues such as nutritional variability, concerns with potential contaminants and impact on feed processing among others. Much of the setting of these 'constraints' derives from issues associated with elements of the risk assessment for use of each raw material.

Defining 'risk'

The process of risk management consists of the systematic application of a series of policies, procedures and practices applied to the tasks of communicating, establishing the context, followed by the identification, analysis, evaluation, treatment, monitoring and review of a given risk, based on a series of considered assumptions and uncertainties (Codex Alimentarius Commission, 2017; Figure 1). Risk assessment is a scientific-based process that is considered to consist of four stages: (i) hazard identification, (ii) hazard characterization, (iii) exposure assessment, and (iv) risk characterization.

Stage 1: Hazard Identification: This stage aims to define the qualitative elements of the adverse consequences associated with a risk, and consolidate the evidence demonstrating that the risk can have an adverse effect (Codex Alimentarius Commission, 2010). For example, in feeds, this might be considering the impact that certain ANF can have on animal performance and this is ratified by drawing from the results of experiments undertaken examining the influence of those specific ANF on animal performance and health.

Stage 2: Dose–response analysis: This stage examines the relationship between the level of exposure to a risk and the probability of the incidence of a certain response/effect (Codex Alimentarius Commission, 2010). This stage is considered to be relatively complex. This complexity is derived

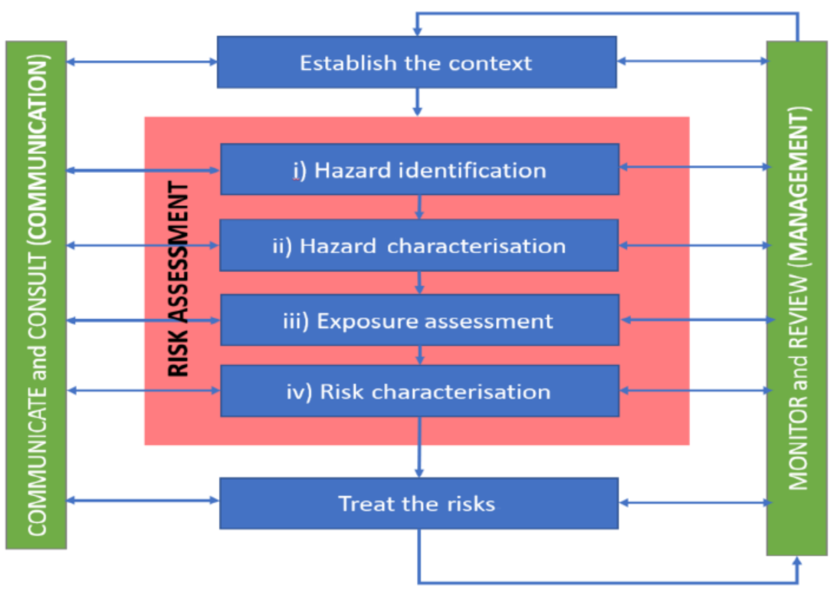

Figure 1 Risk analysis process overview. The steps of risk assessment are highlighted in the red box. Derived from AS/NZS 2004 and Codex Alimentarius Commission, 2010.

from the fact that the assessment of this stage often needs to extrapolate results from *in vivo* experiments across a range of doses and relate those doses with a certain degree of confidence to the observed responses. As with all biological responses, there are often many other factors involved that influence the observed responses, with differences between individuals due to genetics or other factors meaning that the risk may be higher for particular groups (susceptible populations), than others. An alternative to the dose–response strategy is to determine a dose (concentration) where a response is unlikely to result in observable effects. This is regarded as a no effect concentration and has parallels with lethal dose 50% (LD_{50}) studies, albeit looking for no effect within a population rather than a 50% loss in population (Robertson *et al.* 1984; Van der Hoeven 2004). Traditionally, safe dietary levels of feed supplements in toxicological studies are assessed by establishing a no observable adverse effect level (NOAEL) based on a (sub)chronic dose–response study with graded levels of the supplement (Teh *et al.* 2004). The adverse effects assessed are non-lethal adverse effects such as growth or histopathology. The European Food Safety Authority (EFSA), recently evaluated the assessment methodology and proposed the use of the benchmark dose (BMD) model instead of NOAEL to establish safe levels of supplements or contaminants (EFSA, 2017b). In addition, a guidance document was published in which the difference between adverse effect, biomarkers of exposure or effect and mode of action (MOA) were defined (EFSA, 2017a).

Stage 3: Exposure Quantification: This stage aims to determine the level of exposure that individuals and/or populations will receive with the use of a particular risk element (e.g. how much exposure a fish might get to an ANF with the certain inclusion of a raw material) (Codex Alimentarius Commission, 2010). As with the other stages, there are many factors that can influence the amount of each risk element that is exposed, and a range of possible values can often be generated in this stage.

Stage 4: Risk Characterization: The final stage involves the objective (qualitative and/or quantitative) evaluation of the likelihood of a risk occurring and the consequences of that risk arising (Codex Alimentarius Commission, 2010). Part of the difficulty in characterizing risk in an objective manner is that the measurement of both quantifiable elements; that being the potential consequences and probability of likelihood are often very difficult to objectively measure. The chance of error in measuring these two concepts is intrinsically high. Added to this, the responses to risk with a large potential loss and a low probability of occurrence, is often treated very differently from one with a low potential loss and a high likelihood of occurrence.

In terms of the aquaculture feed sector, there are different elements to the risk associated with feed production that need to be considered. First is the risk associated with producing a product (or failing to) to the specifications required for a particular species. In the process of attempting to meet these specifications, risk is encountered in combining raw materials together and the potential for those raw materials to bring in contaminants and pathogens. These contaminants and pathogens can have implications not only for the animal being fed, but also the consumer of that animal.

For the purposes of this review, we are focussing on the first three stages of the assessment: of those risks that impact on the ability to produce an effective feed, the impacts that feed has on the species it is fed to and the potential subsequent impacts that may occur to the human consumer of that animal.

Supply and price

Two critical elements to the viable commercial use of raw materials are their reliable supply and the price for which they are charged. Each of these elements' presents critical risks for feed production.

Supply risk

Most feed manufacturing sites have a finite number of raw material storage options. Because of this constraint, feed manufacturers prefer to allocate those storage options to raw materials that they can routinely and consistently source as it avoids issues associated with mixing and contamination of different raw materials and reduces issues associated with shortfalls in supply of any raw material during the manufacturing process. Therefore, raw materials that are available in large volume are preferential for clear reasons. While small volume raw materials may be options, they are less attractive to manufacturers due to the need to constantly adapt to changing constraints imposed with each new raw material. Consistent changing of raw materials also increases the risks of mistakes being made during the manufacturing process and represents an additional reason why raw materials with large volumes of supply are preferred. However, what constitutes a large (or small) volume supply raw material is a matter of conjecture.

Price risk

Because feeds are clearly made for commercial gain, it is an imperative that there is margin between the raw material costs and sale price of any feed. Like most manufacturing processes, there are a range of economic factors that affect profitability, but key among them is price volatility of various raw materials.

There is substantial variability in price among raw materials. Notably, the price charged for any specific raw material is generally closely linked to their protein and/or profat (protein + fat) content (Figure 2). However, this relationship is not a linear one, with decreasing competition among higher protein content raw materials, there is an increasing price value on these products. In many cases, high protein products are also substantially processed to achieve this degree of protein concentration and this processing comes at a cost (Drew *et al*. 2007a,b).

There is also substantial variability in raw material prices across both spatial and temporal ranges (Figure 3). This in most cases is largely influenced by supply and demand economics. But there are other key factors influencing this variation in the price of specific raw materials, and not all of them respond to the same drivers. It has been suggested that there is growing volatility in global commodity markets due to a shortening of life cycles and economic and competitive forces creating additional uncertainly (Christopher 2000). There are a range of measures that exist to assess volatility in markets, such as the VIX which focusses on a calculation by the Chicago Board of Options Exchange (CBOE) of the stock market volatility for the forthcoming month (30-day period; Whaley 1993;). It has been noted that commodities (raw materials) have more volatility in their price than manufactured products, presumably due to the ability of manufacturers to defray price volatility through varying their raw material use (Jacks *et al*. 2011). However, there are contrasting views that have argued that commodity volatility has not increased over time, and that globalization has reduced volatility and market (economic) isolation has a higher association with commodity price volatility (Jacks *et al*. 2011).

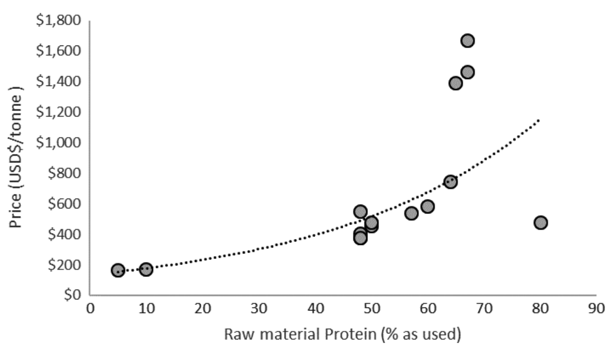

Figure 2 Average price of 14 feed materials over January 2016–December 2016, plotted against their typical protein content. Data derived from www.indexmundi.com

Perhaps, the most obvious link to the cost of production of many of the raw materials used in feeds is the close link to the cost of the energy input into their production processes, generally gauged as the crude oil price, whether that being the cost of operating boats to go to sea to catch fish for fishmeal or the cost of operating farm machinery to grow and harvest crops (Nazlioglu & Soytas 2012; Nazlioglu *et al*. 2013; Mensi *et al*. 2014) (Figure 4a–c). The influence of energy prices on the prices/costs for raw materials can also be seen via the impact of biodiesel and bioethanol on the prices for cereal grains (Serra & Zilberman 2013).

However, since 2006, it has become clear that there has been somewhat of a decoupling of fishmeal and fish oil prices from the crude oil price driver (Olsen & Hasan 2012; Asche *et al*. 2013;Shepherd & Jackson 2013). This has been most likely due to increasing constraints associated with global supplies of these commodities – hence supply and demand economics coming to the fore again (Fig. 3).

Finally, another important factor influencing the volatility of raw material prices is that of the added variable of

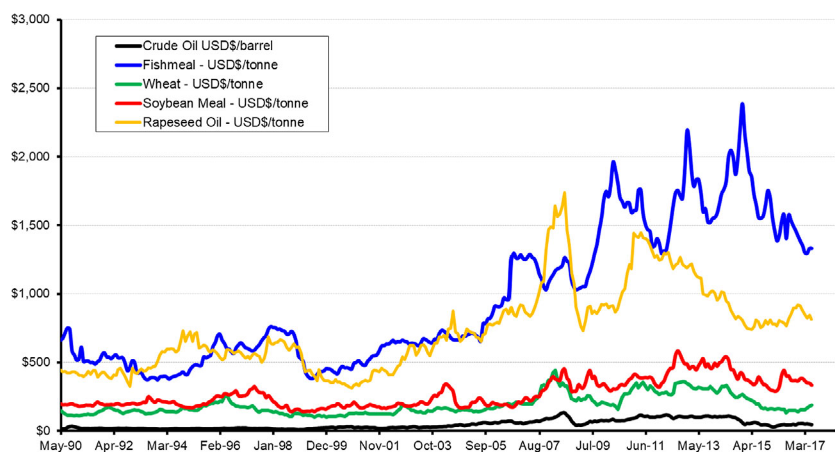

Figure 3 Raw material and resource spot prices from April 1990 to July 2017. Shown are the high degree of volatility in raw material prices and the index of key raw materials relative to others. Data sourced from www.indexmundi.com

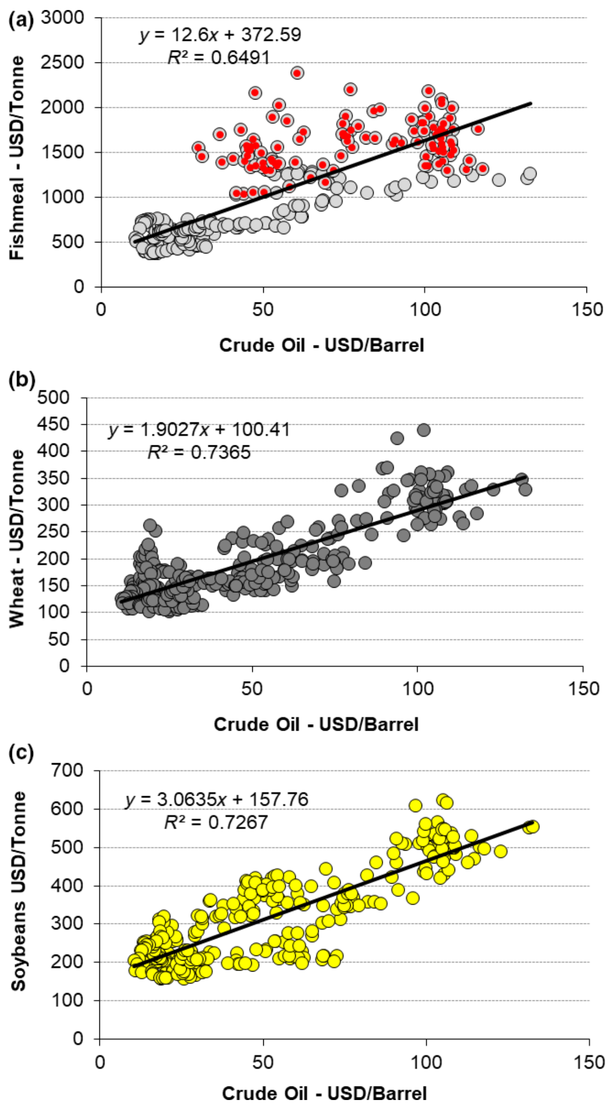

Figure 4 (a, b and c) Correlations between the average monthly price data for key feed raw materials (a. fishmeal, b. wheat and c. soybeans) and crude oil price from 1990 to 2017. Overlaid on the fishmeal data (4a) is the data from 2006 onwards in red. Data sourced from www.indexmundi.com

currency exchange rates (Nazlioglu *et al.* 2013). In global trade, most transactions take place in US$. Therefore, the price paid for internationally sourced raw materials by any manufacturer will be heavily influenced by the rate of currency exchange with whatever the local currency might be. As such, acute changes in foreign exchange rates can also result in acute changes in raw material prices.

Managing supply and price risk

To manage these vagaries in both supply and price risk, there are a suite of strategies that the feed sector traditionally employs. These can generally be grouped as either input related (i.e. linked to raw material acquisition) or process related (i.e. optimizing the use of those raw materials). In terms of process-related controls, the most common is to use linear least-cost formulation, which considers all the different raw materials available against the target product specifications and then optimizes the combination based on meeting those specifications at the lowest cost (Rahman & Bender 1971; Pesti & Miller 1993). A more advanced practice along these lines is to use multimix formulation, which is an extension of the linear least-cost programming approach, that considers many different products at once to optimize the use of raw materials across an entire site or even across an entire business. A variant on this is the use of a multiperiod production plan, which blocks production of products by variation in supply of raw materials according to the most optimal use of those raw materials in the inventory. However, this strategy assumes that in future conditions will improve for production of those products not suited for the raw materials presently in inventory (Applequist *et al.* 2000). A strategy such as this borders on the interaction between the input and process-related controls in feed manufacturing.

A broader approach to reduce the raw material risk is to improve the overall efficiency of the feed production system (Mula *et al.* 2006). In a modern context, two approaches to reduce the risk in manufacturing systems have been considered: 'Lean' and/or 'Agile' manufacturing practices (Hallgren & Olhager 2009). In these practices, the focus has centred more on controlling those internal factors that can be influenced directly by a business to streamline them and as such make the process as 'Lean' as possible. The 'Lean' strategy is simply a method for the elimination of inefficiencies within a manufacturing system, including imbalances in workloads. 'Agile' manufacturing, on the other hand, is a term used to describe an organization that has instigated the processes, tools and training that allows them to respond quickly to changing customer needs and market opportunities, while still maintaining some control over costs and quality. Traditionally, 'Agile' manufacturing is seen as a progression after 'Lean' manufacturing in the evolution of production systems. The major differences in performance outcomes are related to cost and flexibility, such that 'Lean' manufacturing has a significant impact on cost performance (whereas 'Agile' manufacturing has not), and 'Agile' manufacturing has a closer relationship to changes in the volume of production, as well as opportunistic production flexibility, than does 'Lean' manufacturing. A variant on the 'Lean' and 'Agile' concepts has been that the 'Six Sigma' concept, which in essence is a set of techniques and tools for process improvement (Kwak & Anbari 2006). The 'Six Sigma' concept basically seeks to improve the quality of the output of a process by identifying and removing the primary causes of defects and variability in manufacturing and business processes. To do this, 'Six Sigma' uses a series of quality management methods and follows a defined sequence of steps each with specific targets, for example: reduce production time, reduce wastes, reduce costs, increase sales and increase profits.

In terms of raw material input risk management, there are also a series of strategies that can be used. Forward contracting of supplies is one such option, in which a 'forward' contract between a supplier and purchaser is agreed to buy a parcel of a raw material at a specified future time at a pre-agreed price. In such a situation, both parties assume some of the risk by agreeing to the transaction in the future. As the purchaser has to assume a long position (i.e. that the price is likely to go up), the supplier assume a short position (i.e. that the price is likely to go down). Typically, such forward purchasing is not widely used in the aquaculture feed industry, with most companies preferring shorter terms-of-trade for purchasing raw materials (e.g. 90-day terms). Another commonly used strategy is to diversify sourcing options. Essentially, this means ensuring that for any key raw material used, that it is preferably obtained from two or more suppliers. This then allows active competition between the suppliers by keeping the prices down and quality high.

Compositional and nutritional variability

Determining the nutritional value of any raw material is a critical aspect of being able to attribute an economic value to the product (Glencross *et al.* 2007a). However, variability in the nutritional value of any of these products can also impact on their perceived value, with reduced levels of variability being favoured, in that this allows for greater confidence in formulating diets closer to the animal's requirements (Glencross *et al.* 2008a,b). The assessment of variability in the chemical composition of raw materials is one aspect of assessing this nutritional value. This can be readily obtained using standard analytical techniques, although the application of near-infrared (NIR) spectroscopy has led to the development of some rapid (<1 min) assessment systems that allows the cost-effective analysis of large numbers of samples (Aufrere *et al.* 1996). However, a more comprehensive determination of nutritional values and the assessment of their variability have been comparatively more difficult and slower parameter to assess, as it requires information on the extent to which the nutrients from an ingredient are absorbed (digested) and made available for growth (Glencross *et al.* 2014). However, a lack of standardized data on the digestible value of raw materials remains one of the constraints to the broader adoption of many alternative raw materials. Additionally, there is a general paucity of knowledge on the level of intrinsic nutritional variability within many raw materials, with only few studies providing any focus on either rendered animal meals or feed grains (Bureau *et al.* 1999; Glencross *et al.* 2008a). Furthermore, the effective characterization of this variability and, just as important, the characterization of the origins of the raw materials being assessed (e.g. where it was produced, how it was processed, etc.) are key issues that need addressing to enable firstly an understanding of the extent of the problem and then secondly to empower research to provide solutions.

Causes of variability

Variability exists in all raw materials. For feed grains, there are numerous causes of this variability. Protein, carbohydrate and lipid levels in all feed grains can vary considerably depending on growing season attributes, cultivar, farm management practices and soil conditions (Longnecker *et al.* 1998; Peterson *et al.* 1992; Glencross *et al.* 2008a). In addition to these primary production points of control, subsequent management of feed grains can also impart significant variability to their nutritional value. Differences in segregation, storage and processing have also all been implicated in affecting the feed grain composition. Importantly, such variability in composition has also been noted to extend to the digestible value of feed grains and other raw materials and occurs across species (Glencross *et al.* 2008a, 2017, 2018;Tabrett *et al.* 2012; Ngo *et al.* 2015).

Similarly, rendered animal products can also be quite variable and this variability has been implicated as one of the key reasons limiting their application in aquaculture feeds (Bureau *et al.* 1999). Points of influence in rendered products include the animal species used, what components are included (e.g. whole animal, deboned, bone-in, blood, etc.), age of the components since slaughter, temperature of storage of the components (e.g. chilled or ambient), cooking temperature during wet rendering and the drying method employed. There is evidence to support that each of these control points in the rendering process can affect the nutrient composition and nutrient digestibility of rendered animal products (Bureau *et al.* 1999; Glencross *et al.* 2017, 2018).

The variability in the nutritional value of a raw material depends on both the total nutrient content and the biological availability of the specific nutrients it contains (Jiang 2001). This biological availability has two aspects to it: the ability of an animal to absorb nutrients (digestibility) from the raw material and the ability of the animal to convert those nutrients into growth (utilization) (Glencross *et al.* 2007a).

Implications of variability

The nutritional value of most feed grains is usually a direct reflection of their digestible nutrient (and energy) content

(Glencross *et al.* 2004a; Aslaksen *et al.* 2007). Accordingly, any variability in the digestible value of these raw materials should translate to variability in their economic value. Arguably, the combination of compositional variability with digestible variability means that the true economic value of raw materials is actually much wider than given credit for (Glencross *et al.* 2018). Furthermore, the combination of variability in crude composition and that of the digestible value is compounded, with the resultant impact of substantially greater variability being observed in the actual levels of digestible nutrients (Glencross *et al.* 2008a, 2018). In a study examining the nutritional value of lupin meals, there was an exacerbated level of variability observed in both the values of digestible protein (coefficient of variation of 11.3%) and digestible energy (coefficient of variation of 8.2%), which were greater than that of the variability in both the compositional variability (7.6% and 1.5% for protein and energy respectively) and digestibility (10.3% and 8.0% for protein and energy digestibility, respectively; Glencross *et al.* 2008a).

In addition to the variability in composition and digestibility of raw materials, the consequences of not effectively managing this has been demonstrated in terms of a direct and measurable impact on their nutritional value. In a series of studies where the diets were formulated on their gross compositional values, it was possible to demonstrate the direct impact associated with variability in the digestibility of protein and energy from a single component raw material in those diets (Glencross *et al.* 2008b). However, in the process of assessing this variability, it also became possible to identify those compositional features of feed grains that contributed to not only variation in the composition, but also the inherent digestibility of the raw materials themselves (Glencross *et al.* 2008a). This has since been followed up by a series of manipulative trials to focus on those specific non-starch polysaccharides (NSP) that influence this process of digestibility the most (Glencross *et al.* 2012; Irvin *et al.* 2016). This ability to chemically identify those factors within raw materials that affects their own nutrient and energy digestible values lends itself to development of further raw material assessment methods, such as the use of NIR to measure digestibility of both individual raw material and compound diets (Glencross *et al.*, 2014; Glencross *et al.* 2016).

Strategies to manage variability

There are a range of strategies that can be employed to manage raw material variability. Typically, this variability is managed, to an extent, through increasing the diet formulation specifications to allow for an over-specification of key nutrients. Although this formulation strategy reduces performance risk, it does increase the cost of the diet manufacturing process. The capacity to better manage this variability depends on an improved ability to rapidly measure the nutritional value of raw materials prior to the formulation process and an ability to capture and respond to the information in near real-time (Jiang 2001). There are several options that can be considered for managing such raw material variability, but ultimately it is probably the adaptation of the use of NIR spectroscopy that is one of the more viable options to pursue for such near real-time adaptation and this will be discussed later. However, there are other options by which the inherent variability in raw materials can be managed.

Bulking and blending

One common strategy, and perhaps arguably, the most common one used prior to raw materials arriving at a feed mill, is their large-scale bulking and blending through bulk receival. In the cases of grains, these are usually received from growers at centralized receival and bulk storage points nearby the grain production regions. This pooling of material has the propensity of averaging out the composition across the pooled materials. This practice, while having the advantage of homogenizing materials to some extent, does also diminish the value of those materials of higher value by subsidizing those materials of lower value. A practice becoming increasingly common among grain growers is the self-segregation of crops on-farm that are of higher value. In this way, some farmers are assessing their best options for their produce prior to sale in an attempt to garner the greatest margin. Such a practice also has benefits for the feed producer, provided responses can be made to adapt to those better qualities (e.g. higher protein and fat levels) before the raw material is used.

Processing

Another raw material management option is to process the raw materials to minimize their variability, and in many cases also maximize their nutritional value. There is a wide range of processing strategies that can be applied here, from the dehulling of grain (removal of the fibrous seed coat), to the dewatering of blood to produce a high-protein dry powder (Drew *et al.* 2007a). Other strategies can involve the use of heat to reduce the influence of ANF or the inclusion of exogenous enzymes to reduce their effects (Drew *et al.* 2007a; Lin *et al.* 2007). The advantage of processing raw materials is in the capacity to improve their value as a feed constituent. In addition, the inherent variability can be influenced, but it must be acknowledged that processing can also increase this variability in some instances subject to the processes used and their levels of efficiency in changing the composition of the raw material. In most legume feed grains (soybeans, faba beans, field peas and lupins) an increase in protein concentration is typically reciprocated by a decrease in the levels of NSP (Glencross *et al.* 2007b, 2008b). This processing has additional merits above just increasing the protein levels in the raw material, as high levels of some types of NSP have been implicated in lower nutritional value of the raw materials they are within (Glencross 2009; Irvin *et al.* 2016). The level of lignin in particular has been implicated as a negative factor in protein digestibility via both multivariate analysis and empirical means (Glencross *et al.* 2008b, 2012; Irvin *et al.* 2016). With raw materials derived from animal sources, drying the material by heating is perhaps the most common processing method used. Heating, while useful in removing water and improving the microbiological stability of a raw material, it can also impart damage through a range of chemical reactions including Mailliard reactions, disulfide-linkages and burning (Oste 1984). Increasing levels of heat imparted in the drying process have been implicated in a reduction in the nutritional value of some raw materials (Bureau *et al.* 1999; Glencross *et al.* 2004b; El-Haroun & Bureau 2007; El-Haroun *et al.* 2009).

Rapid analysis technologies

The development of technologies for the rapid analysis of nutritional value of raw materials, such as the use of *in vitro* assays and scanning technologies, like NIR, have been the subject of research since the 1980s (Eid & Matty 1989; Dimes & Haard 1994; Bassompierre *et al.* 1997; Carter *et al.* 1999; Tibbetts *et al.* 2011a,b; Wrigley 1999). A range of *in vitro* methods have been examined in terms of their utility in providing estimates of the nutritional (digestible) value of different raw materials (Eid & Matty 1989; Bassompierre *et al.* 1997). Among the different methods examined, they are generally consistent in using an enzyme mediated process, but it is often what enzymes are used (purified preparations or crude homogenates) and how the resultant products of the enzymatic process are used and assessed that vary. A key component to the viable use of any rapid assessment method must be its validation against *in vivo* methods of assessment, as these are the primary responses that are being sought to be replaced (Dimes & Haard 1994). Despite considerable effort being spent on developing and testing a range of *in vitro* methods, it has been stated that they are still time-consuming and have problems surrounding their reliability and inconsistencies in their predictive ability (Bassompierre *et al.* 1997). The comparison of a rainbow trout pyloric caeca homogenate (and various subfractions) *in vitro* assay (pH-stat) method against that of *in vivo* digestibility was reported by Dimes and Haard (1994). These authors reported correlations ranging from 0.17 to 0.87. The validation of a series of *in vitro* assays using purified preparations of trypsin (porcine), chymotrypsin (bovine) and protease (bovine) or homogenated extracts of Atlantic salmon pyloric caeca were compared against the *in vivo* data from the same eight diets (Carter *et al.* 1999). For either method, the correlation between the *in vitro* and *in vivo* data was poor ($R^2 < 0.2$). Subsequently, modifications have been made to various *in vitro* assays and improvements to the correlation between the *in vivo* apparent digestibility data with a range of species and *in vitro* degree of hydrolysis data have been reported, with the R^2 values being as high as 0.99 (Tibbetts *et al.* 2011a,b; Yasumaru & Lemos 2014). While the use of *in vitro* technologies has not been overly successful in terms of routine adoption by industry, the advent of NIR has perhaps been one of the landmark progressions in the management of raw material variability (van Barneveld *et al.* 1998; Glencross *et al.*, 2015).

In contrast to *in vitro* assays, technologies like NIR and nuclear magnetic resonance (NMR) spectroscopy have allowed the assessment of the nutritional value of raw materials, on a near real-time basis, and provide significant advancements in the responsiveness and cost savings in diet formulation by the aquaculture feed industry (Conceição *et al.* 2003; Pujol *et al.* 2007; Glencross *et al.*, 2015). The use of NIR for determining the composition of raw materials is now relatively common in most modern feed production systems throughout the world. However, the use of NIR to assess the digestible value of protein and energy from raw materials is not well established and reports on its successful application to aquaculture species are scarce (Glencross *et al.*, 2015). To achieve a viable NIR calibration, it is critical that a wide range of samples is obtained from which to determine the nutritional (digestible protein and energy) values of the raw materials and to then correlate this with the NIR spectra of those same samples (van Barneveld *et al.* 1998; Glencross *et al.*, 2015; Glencross *et al.* 2016). The process of calibration development can be laborious and costly, although the potential gains in functionality through this method are enormous.

Contaminants

Like all biological products, plant and animal protein meals (and oils) can suffer from contamination with chemicals. In general, contamination of feed ingredients can occur on an unintended basis by the presence of undesirable environmental contaminants such as metals or persistent organic pollutants (POPs; e.g. dioxins, polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs), brominated flame retardants (BFRs)), or by the treatment of raw material/crop such as the use of pesticides or heat processing. In addition, feed ingredients can be contaminated by natural toxins such as mycotoxins that are produced by fungi. Residues of metals, POPs, pesticides and mycotoxins can contaminate meals and oils causing a significant reduction in their nutritional value (van Barneveld 1999) and potentially form a risk for fish health and/or food safety. In the next section of this review, we have taken the approach of attempting to explore the impacts of these different classes of contaminants on fish and where possible examine their dose–response, histological, enzymatic and gene expression effects (toxicodynamics). Additionally, the kinetics of both accumulation and depletion have also been examined.

Environmental persistent organic pollutants

Persistent organic pollutants that are of importance for aquafeeds and farmed fish include dioxins, dioxin-like polychlorinated biphenyls (DL-PCBs), non-dioxin-like PCBs, BFRs, and OCPs such as dichlorodiphenyltrichlorethane (DDT), hexacyclobenzene (HCB), toxaphene, aldrin, chlordane, endosulfan and hexacyclohexane (HCH). These POPs are all halogenated (e.g. contain chlorine and bromine) carbon structures, which can have many different chemical isoforms (congeners) (Safe *et al.* 1985). In 2001, the United Nations Environment Program (UNEP) put into practice the Stockholm Convention, which recognized the potential human and environmental toxicity of a suite of POPs and listed 12 particular POPs as their 'dirty dozen' (Table 1). The listed 12 compounds were noted as being particularly potentially harmful compounds that needed to be addressed globally for the future, with action required by the convention signatories to eliminate or reduce the release of these compounds to the environment. Since the original 'dirty dozen' were defined in 2001, another 10 have been added to the list in later years. The levels of POPs are subject to a global treaty, the Stockholm Convention, that aims to restrict and eliminate production and use of 12 major POPs and has been ratified by 150 countries and written into EU legislation under Regulation No 850/2004 (European Commission, 2004). The chemical structure of these environmental pollutants, containing halogenated benzene rings, means they are lipophilic and resistant to degradation and bioaccumulate in food chains, particularly in the marine environment (Gobas *et al.* 1999). Therefore, marine feed ingredients particularly fish oils and, to a lesser extent, fishmeal, derived from pelagic fisheries are the main sources of POPs in farmed fish (Easton *et al.* 2002; Jacobs *et al.* 2002a,b). The POP levels in fish oils depend on several factors including season, fish species, age and geographical origin (Bell & Waagbø 2008; NORA 2003). Fish oils from the Pacific Ocean generally have lower levels of dioxin and, to a lesser degree, PCBs than fish oils from the Atlantic Ocean (Lundebye *et al.* 2004; Berntssen *et al.* 2005; Kelly *et al.* 2008). The Baltic Sea is a well-known polluted area and fish oil derived from pelagic fish from the Baltic have high levels of POPs with dioxins and DL-PCBs (see under) with levels exceeding upper limits (Lundebye *et al.* 2004). Fish oil from pelagic fish species caught in the North Atlantic Ocean in winter have considerably lower levels of dioxins and PCBs than fish oils obtained from fish caught in the spring. During early spring the lipid content decreases in the fish and consequently the concentration of POP increases in the extracted oil (NORA (Nordisk Atlantsamarbejde) 2003).

Dioxins and dioxin-like compounds (DLCs)

One major group of contaminants commonly associated with raw materials for fish feeds are the dioxins and dioxin-like compounds (DLC) that are by-products of many industrial as well as some natural processes such as forest fires. Dioxin is a generic term given to two chlorinated ground structures namely polychlorinated dibenzo-*p*-dioxins (PCDD) and polychlorinated dibenzofurans (PCDF). DLC includes DL-PCBs that have the same toxic mechanism as PCDD/Fs. There are 75 PCDD, 135 PCDF and 130 PCB 'congeners' that differ depending on the number and position of the chlorines with 7 PCDDs, 10 PCDFs and 12 PCBs (DL-PCBs) being regarded as toxic (Van den Berg *et al.* 1998, 2006). Toxic DLCs are teratogenic, mutagenic, carcinogenic, immunotoxic and hepatotoxic with toxicity based on interaction with the aryl hydrocarbon receptor (AhR), a transcription factor that affects many regulatory pathways. The most toxic and well-studied dioxin congener is 2,3,7,8-tetrachlorodibenzodioxin (TCDD) and the toxicity of all other dioxin and DLC congeners are measured in relation to this and assigned a Toxic Equivalence Factor (TEF) from 0 to 1 (TCDD = 1) (Ahlborg *et al.* 1994; Van den Berg *et al.* 2006; Tuomisto 2012), with the toxicity of mixtures of dioxins and DLCs, as found in feed ingredients, expressed as total dioxin equivalents (TEQ). The health risks of dioxins and DLCs have been assessed by the Food and Agriculture Organization of the United Nations (FAO) and World Health Organization (WHO) Expert Committee on Food Additives (JECFA)(FAO/WHO, 2002, 2004, 2007). The former European Commission (EC)'s Scientific Committee for Food established a tolerable weekly intake (TWI) in 2001 of 14 pg WHO-TEQ/kg body weight (bw) for dioxins and DL-PCBs and the expert committee of European food safety authorities has currently re-assessed this upper limit.

The European Union (EU) has established maximum permitted levels for dioxins (17 PCDD/PCDF congeners) and DL-PCBs (12 congeners) in both animal feed and food for humans. The EU maximum residue level (MRL) for the sum of dioxins (PCDD/PCDF) in the muscle meat of fish and fishery products and products thereof, with the exemption of eel, fish liver and marine oils, is 3.5 pg WHO-TEQ/g fresh weight, and the EU maximum level for the sum of dioxins and DL-PCBs is 6.5 pg WHO-TEQ/g. For eels, the

Table 1 The original 'dirty dozen' persistent organic pollutant compounds on the Stockholm Convention list

Compound	Use	Source	Half-life	Impacts on animals and humans
Aldrin	Insecticide	Dairy and meat	5 years	•Fish – LD_{50} of 0.01 mg/kg •Possible carcinogen
Chlordane	Insecticide	Air pollution	365 days	•Compromises immune system •Possible carcinogen
Dieldrin	Insecticide	Human exposure occurs primarily through food	5 years	•Linked to Parkinson's disease, breast cancer, and classified as immunotoxic, neurotoxic, with endocrine-disrupting capacity •Highly toxic to fish and other aquatic animals
Endrin	Insecticide/ Rodenticide	Human exposure occurs primarily through food	12 years	•Endrin is highly toxic to aquatic animals and humans as a neurotoxin
Heptachlor	Insecticide	Human exposure occurs primarily through food	250 days	•Laboratory tests have shown high-dose is lethal, with adverse behavioural changes and reduced reproductive success at low-doses •Possible human carcinogen
Hexachlorobenzene (HCB)	Fungicide	Human exposure occurs primarily through food	6 years	•Photosensitive skin lesions, colic, debilitation, and a metabolic disorder called porphyria turcica, which can be lethal •Mothers who pass HCB to their infants through the placenta and breast milk had limited reproductive success including infant death
Mirex	Insecticide/Flame retardant	Human exposure occurs primarily through food	10 years	•Mirex is toxic to several plant, fish and crustacean species, with suggested carcinogenic capacity in humans
Toxaphene	Insecticide	Human exposure occurs primarily through food	12 years	•Toxaphene is highly toxic to fish, inducing dramatic weight loss and reduced egg viability •While human toxicity to direct toxaphene exposure is low, the compound is classified as a possible human carcinogen
Polychlorinated biphenyls (PCBs)	Used as heat exchange fluids, in electrical transformers, and capacitors, and as additives in paint, carbonless copy paper, and plastics	Human exposure occurs primarily through food	10 years, though persistence varies with degree of halogenation	•Toxic to fish at high doses, and associated with spawning failure at low doses •Associated with reproductive failure and immune suppression •Immediate effects of PCB exposure include pigmentation of nails and mucous membranes and swelling of the eyelids, along with fatigue, nausea and vomiting •Effects are transgenerational, as the chemical can persist in a mother's body for up to 7 years, resulting in developmental delays and behavioural problems in progeny
Dichlorodiphenyl-trichloroethane (DDT)	Insecticide	Human exposure occurs primarily through food	10–15 years	•DDT is toxic to many organisms including birds where it is detrimental to reproduction due to eggshell thinning •Short-term acute effects of DDT on humans are limited •Long-term exposure has been associated with chronic health effects such as diabetes, carcinogenic, reduced reproductive success, and has been linked to neurological disease
Dioxins	By-products of high-temperature processes, such as incomplete combustion and pesticide production	Human exposure occurs primarily through food	9–100 years	•Humans immune and enzyme disorders •Possible human carcinogen •In laboratory studies result in an increase in birth defects and stillbirths •Lethal exposure has been associated with the substances

(Continues)

Table 1 (continued)

Compound	Use	Source	Half-life	Impacts on animals and humans
Polychlorinated dibenzofurans	By-products of high-temperature processes, such as incomplete combustion and pesticide production.	Human exposure occurs primarily through food	9 years	•Structurally similar to dioxins, the two compounds share toxic effects •Possible human carcinogens

maximum levels for the sum of PCDD/PCDF and the sum of PCDD/PCDF/DL-PCB are 3.5 and 10 pg WHO-TEQ/g, respectively (EC Regulation No 1259/2011) amending Regulation (EC) No 1881/2006 as regards maximum levels for dioxins, DL-PCBs and non-DL-PCBs in foodstuffs). For fish oil intended for animal feed, the levels are 5.0 pg dioxins (TEQ-WHO)/g and 20.0 pg dioxins plus DL-PCBs (TEQ-WHO)/g, and in feed 1.75 pg dioxins (TEQ-WHO)/g and 5.5 pg dioxins plus DL-PCBs (TEQ-WHO)/g. Similar regulatory guidelines have been produced by other national food safety agencies (e.g. Canadian FIA, 2005; USFDA, 2006). Due to the lipophilic nature of POPs, they accumulate in the fat components of fish and so oily fish fed on high energy diets containing up to 38% fat such as the salmonids, especially Atlantic salmon, have attracted some attention. Reports of the levels of dioxins, DL-PCBs and PBDEs in salmon, especially farmed salmon (Hites *et al.* 2004a,b) and salmonid feeds (Foran *et al.* 2005; Maule *et al.* 2007; Kelly *et al.* 2008) prompted concern that led to decreased salmon sales in the US. A panel of experts convened by the EFSA to address the issue reached the conclusion that there was insufficient difference in contaminant levels between wild and farmed salmon to differentiate risks to human health (EFSA, 2005). Several subsequent studies applied well considered risk–benefit analyses and concluded that the health benefits of consuming fish and seafood outweighed by at least 100-fold the perceived health risks, which may not exist at all (Rembold 2004; Tuomisto *et al.* 2004; Cohen *et al.* 2005; Mozaffarian & Rimm 2006; FAO/WHO, 2011;).

The OCPs are another group of POPs for which the EC has established maximum permitted levels in feed ingredient, animal feeds (EC 2005) and food products for human consumption. As PCBs and dioxins, these pesticides are chlorinated hydrocarbons, or organochlorines, and several classes of these pesticides exist such as those initially included in the Stockholm Convention: aldrin, dieldrin, chlordane, DDT, heptachlor, hexabenzene, mirex and toxaphene. These OCP pesticides have mostly been banned for agricultural use (Magulova & Priceputu 2016), and have been replaced by less persistent and more water-soluble pesticides that have lower potential than OCPs to bioaccumulate in the aquatic ecosystem (Seiber 2002).

The flame-retardant chemical, polybrominated diphenyl ethers (PBDEs), are as PCBs and dioxins halogenated carbon structures except that chlorine is replaced by bromine. In contrast, PBDEs do not exert Ah-like properties and are not considered genotoxic or carcinogenic but rather affect thyroid hormones and cause hepatic and thyroidal histopathological changes resulting in neurotoxicological and behavioural effects (FAO/WHO 2002, 2004, 2007). As opposed for the OCPs and dioxins and DL-PCB, no EU upper limits for PBDE in feed ingredients exists yet. Further flame retardants including polybrominated biphenyls (PBBs), hexabromocyclododecane (HBCD) and tetrabromobisphenol A (TBBPA) for which no EU legislation on feed ingredients exist yet.

In addition, as POPs are associated primarily with the use of fish oil and fishmeal and proportions of these marine ingredients in feeds for farmed fish are necessarily declining due to their finite and limiting nature of their supply (Tocher 2015), the perceived problem is also decreasing. For example, in Norwegian salmon feeds from 1990 to 2013, the proportions of marine ingredients decreased from almost 90% down to about 29%, with plant ingredients (other than starch) increasing from zero to almost 56% of feeds (Ytrestøyl *et al.* 2015). There has been a progressive decline in the levels of POPs in 2000 (65% marine and 22% plant) and 2010 (42% marine and 48% plant). Several studies have demonstrated how replacement of fish oil with vegetable oils reduced the levels of POPs in farmed salmon (Bell *et al.* 2005, 2008; Berntssen *et al.* 2005, 2010a, 2011; Drew *et al.* 2007b; Friesen *et al.* 2008; Pratoomyot *et al.* 2008; Sprague *et al.* 2010). Therefore, because of the decreased use of marine ingredients, the presence of these POPs in both feeds (Sissener *et al.* 2013) and farmed salmon (Nøstbakken *et al.* 2015) has been decreasing and consequently so is the risk associated with these contaminants. A recent comprehensive report from the Norwegian Scientific Committee for Food Safety (VKM) concluded that, with the present mean level of dioxins and DL-PCBs in fish on the Norwegian market, even for those with high fish consumption, the exposure to dioxins and DL-PCBs from fish represented negligible risk and was of no concern (VKM, 2014). Furthermore, the changing feed ingredient base has resulted in farmed salmon now generally have lower contaminants levels than wild salmon, at least in Europe (EFSA, 2012). In addition, a recent study of consumer beliefs in Europe found that, in general, farmed fish was perceived to be less affected by marine pollution than wild fish (Claret *et al.* 2014).

Nevertheless, it should be acknowledged that replacement of marine ingredients with plant ingredients can increase the load of other contaminants such as non-OCP pesticides that are currently used on crops, and increased occurrence of these pesticides have been reported for plant-based replacement feed (Nácher-Mestre *et al.* 2014). The EU MRL legislation for non-OCP pesticides comprises most food commodities (European Commission, 2005), but for feed ingredients (crops exclusively used for animal feed purposes) and fish, harmonized EU MRLs are not yet established. In 2013, crops used as a feed ingredient and fish were added as commodity categories with no set MRL yet (European Commission, 2013). Unrefined plant oils obtained from oilseeds such as soybeans, rapeseeds, olive seeds and sunflower seeds are known to contain elevated levels of polyaromatic hydrocarbons (PAHs). The replacement of marine ingredients with plant feed ingredients, and in particular plant oils, gives an increase in PAH in salmon feeds (Berntssen *et al.* 2015). Although there is EU legislation on PAHs in food products, similar to the non-OCP pesticides, no legislation with respect to feed ingredients exists.

In the longer term, the risk associated with marine ingredients is also likely to decrease as levels of POPs in the environment are already generally decreasing (Bignert *et al.* 1998) and should continue to do so due to the Stockholm Treaty eliminating production and use of these POPs. However, due to their persistent nature, it will be some time before levels would be low enough to make monitoring unnecessary. In the meantime, in addition to the replacement of marine ingredients with plant ingredients, decontamination of fish oils is another strategy to reduce contaminant loads from feed ingredients (Breivik & Thorstad 2005; Oterhals *et al.* 2007). This is necessary for some Baltic fish oils that have contaminant levels that exceed the EU limits and so must be decontaminated before they can be used in feeds. Activated carbon has traditionally been the most commonly used in method for effectively removing PCDDs and PCDFs but it is less effective for the removal of DL-PCBs or PBDEs (Maes *et al.* 2005; Oterhals *et al.* 2007). In contrast, volatilization techniques are more efficient in removing DL-PCBs but less effective in removing PCDDs and PCDFs (Carbonelle *et al.* 2006). Therefore, a combination of decontamination techniques is required to effectively remove all these POP groups (Breivik & Thorstad 2005; Carbonelle *et al.* 2006; Oterhals *et al.* 2007; Kawashima *et al.* 2009). Depending on the experimental conditions, short path distillation can potentially reduce the levels of lipid soluble nutrient such as vitamin D and E, but to a far lesser extent that the removal of POPs (Berntssen *et al.* 2006). The use of decontaminated fish oil in feeds has been demonstrated to reduce contaminant levels in farmed salmon without any apparent detrimental effects on fish performance or health suggesting nutrient levels were not substantially affected by the decontamination processes (Bell *et al.* 2008; Pratoomyot *et al.* 2008; Berntssen *et al.* 2010b; Olli *et al.* 2010; Sprague *et al.* 2010). Therefore, decontamination of fish oil could be a useful strategy, especially for highly contaminated oils such as from the Baltic fish, and particularly if refining processes continue to advance. This would enable the safety of farmed fish to be ensured and allow the considerable health benefits associated with n-3 long-chain polyunsaturated fatty acids to be obtained by human consumers without concerns over POPs (Lall 2010). Recently, it was announced that Marine Harvest had entered into an agreement with FF Skagen to clean all relevant fish oil used for Marine Harvest salmon farming (Marine Harvest, 2014).

Heavy metals and radionuclides

Metals and metalloids can exert toxic actions to most organisms when present at levels exceeding their natural trace background levels. In general, the metals and metalloids can be divided into essential and non-essential elements. The essential elements include those metals and metalloids such as copper (Cu), zinc (Zn), iron (Fe), manganese (Mn), and selenium (Se). However, even essential dietary elements can be toxic at high dietary intake levels. Potential toxic effects to farmed fish from ingestion of essential elements can occur during environmental pollution (e.g. water pollution), or in particular to farmed animals, through contamination of a feed ingredient or the over supplementation in fish feeds as part of the minerals mix. Possible adverse effects and some threshold limits for farmed fish have been reviewed earlier by (Baeverfjord *et al.* 2018).

Chief among the non-essential metals and metalloids of concern for animal health and food safety are arsenic, cadmium, lead and mercury (Neathery & Miller 1975). Legislation regulating the levels of such undesirable substances in foods and feeds is usually based on the total concentration. However, the toxicity of metals is highly dependent on their chemical form. The chemical form of the metals and metalloids is of importance for its ability to contaminate edible parts of the finfish as well as potential toxicity to the consumer. Both mercury and arsenic are mainly present in an organic form in finfish samples (Shiomi *et al.* 1995; Francesconi & Edmonds 1996; Amlund & Berntssen 2004; Amlund *et al.* 2006, 2007). Organic forms accumulate more readily in the aquatic food chain and fish muscle during farming than the inorganic forms. Whereas the organic form of arsenic in finfish (arsenobetaine) is non-toxic compared to the inorganic arsenic form, for mercury the organic form is more toxic than the inorganic form (Berntssen *et al.* 2017). This makes the organic form of mercury, methylmercury, the most important metal with regards to food safety and potential adverse effect on the farmed fish.

Decontamination of substances containing metals is distinctly different from that of POPs. Metals and metalloids cannot be removed using common POP decontamination processes. The most common manner of metal management is via dilution and maximum residue levels (MRL) (Nasreddine & Parent-Massin 2002). Some feed raw materials in particular tend to be markedly higher sources of heavy metals, like fish and krill meals (Berntssen *et al.* 2010a). As such, studies have shown that the increased use of alternative protein sources in fish feeds can lead to a reduction in the level of heavy metals in fish and fish muscle (Berntssen *et al.* 2010a).

Another aspect to metal toxicity is that of radioactive metals. Metals can have both radiological toxicity and chemical toxicity and the former will be discussed later. Toxicity studies on some key heavy metals and their organic compounds have been considered very important, particularly so in terms of their effects on the aquatic environments where they tend to accumulate. As such, most studies assess the uptake of these contaminants via passive uptake (gills and food-chain accumulation), and less so via direct active (consumption) uptake. In this section, we have attempted to review those studies that have focussed on active uptake via the diet and when possible with a focus on the use of aquaculture related species.

Arsenic

The metalloid arsenic (atomic number 33, atomic weight 74.9) is reported to induce poisoning (Hughes 2002). Arsenic compounds have many properties similar to that phosphorus. Arsenic usually has an oxidation state of –3 in the arsenides and + 3 in the arsenites, arsenates and organoarsenic compounds. The organic arsenic forms are the dominant forms present in fish (Sloth *et al.* 2005) and can be present as both the form of lipid-soluble (Sele *et al.* 2012) and water-soluble compounds (Sele *et al.* 2015). The most common organic form of arsenic is arsenobetaine, which is considered to be non-toxic (Amlund *et al.* 2006). However, it is the inorganic compounds that are considered poisons and have been widely used as insecticides (Smedley & Kinniburgh 2002). When consumed by humans, arsenic leads to brain damage, compromises the immune system and is also a carcinogen. The oral toxicity (LD_{50}) for a mouse is ~150 mg/kg, though this does vary with form (Hughes 2002). At a biological level, arsenic interferes with ATP production from the TCA cycle and it also uncouples oxidative phosphorylation. It has also been linked to an increase in hydrogen peroxide production resulting in an increased production of reactive oxygen species and subsequently exacerbating oxidative stress (Hughes 2002).

Organoarsenic in fish is generally considered to be derived from lower stages of the marine food chain (Hanaoka *et al.* 1992). Higher levels of arsenic have been observed in fish fed diets based on marine raw materials than those fed diets based on terrestrial derived raw materials (Hanaoka *et al.* 1992; Berntssen *et al.* 2010a). Analysis of samples of feeds for salmon and fishmeals from the Norwegian Fish Feed Monitoring Programme in 2003 were analysed for their total arsenic and inorganic arsenic contents (Sloth *et al.* 2005). Concentrations in the ranges of 3.4–8.3 and 0.010–0.061 mg/kg in feeds were found for total arsenic and inorganic arsenic respectively. Several of the feed samples had total arsenic concentrations above the EU maximum content of 6 mg/kg for complete feeds for fish. However, the levels of inorganic arsenic, constituted less than 1.2% of the total arsenic content. In a more recent study, Berntssen *et al.* (2010a) also found that inorganic arsenic comprised only a small (< 2%) fraction of the total arsenic content.

There are a series of organic forms of arsenic known as arsenolipids (Sele *et al.* 2012). Little is known about the chemistry and potential toxicity of these lipid-soluble forms of arsenic. Lipid-soluble organoarsenic compounds have a similar biological half-life as water-soluble ones of about 50 days (Hanaoka *et al.* 1992). Of those organoarsenic compounds that form lipids, the majority end up in the polar lipid fraction.

Inorganic forms of arsenic are the most toxic (Amlund & Berntssen 2004). The absorption of arsenic is influenced by the concentration of the compounds present in the diet (Hanaoka *et al.* 1992). The uptake of a labelled source of dietary inorganic arsenic is dependent on the concentration of arsenic in the animal, suggesting that first-order kinetic processes are involved. However, only a small percentage of the inorganic arsenic is converted into any of the organic forms. Organic arsenic readily accumulates in the muscle of fish, whereas the toxic inorganic form accumulates mostly in the viscera (Amlund & Berntssen 2004).

Cadmium

Cadmium (Cd) is a non-essential heavy metal that in fish is bound to proteins that contain considerable numbers of sulfhydryl groups (SH). Cadmium occurs naturally in the environment as a result of volcanic emissions. In addition, anthropogenic activity (e.g. use in battery pigments) has increased the background levels of Cd in soil, water and organisms. Cadmium is not a transitional element and unlike essential elements such as copper and manganese, and does not exert redox properties itself (Nebergall *et al.* 1968). Nevertheless, oxidative stress has been observed in Cd-exposed mammals(Ognjanovic *et al.* 2008) and fish (Berntssen *et al.* 2000). Cadmium exposure has been suggested to provoke oxidative stress through impairment of, among a series of enzymes, the endogenous antioxidant enzymes that are rich in SH groups such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) (Ognjanovic *et al.* 2008). Cadmium-induced inhibition of these enzymes will give rise to the formation of reactive oxygen radicals (Ognjanovic *et al.* 2008). Alternatively, Cd can provoke the activation of the cytochrome P450 detoxifying system by forming carbon-centred radicals such as trichloromethyl radicals (CCl_3) which rapidly react with O_2 to give peroxyl radicals (O_2CCl_3) and initiate oxidative stress(Halliwell & Gutteridge 1993). In biological situations, this increases lipid peroxidation, depletes antioxidants, glutathione and protein-bound sulfhydryl groups and promotes the production of inflammatory cytokines (Kayama *et al.* 1995).

There have been several studies examining the exposure of a range of species of fish to feed containing Cd (Pratap & Bonga 1993; Lundebye *et al.* 1999; Franklin *et al.* 2005; Dang & Wang 2009). Berntssen *et al.* (2001) examined the response of Atlantic salmon (*Salmo salar*) parr to exposure of one of six dietary Cd concentrations (0, 0.5, 5, 25, 125 or 250 mg/kg) for 4 months. These authors observed that the rates of apoptosis and cell proliferation in the intestine increased following exposure to dietary Cd. However, the exposure to elevated concentrations of dietary Cd had no effect on growth or development in the fish Lundebye *et al.* (1999). The effects of both dietary and water-soluble Cd on the gills of the African freshwater cichlid (*Oreochromis mossambicus*) were examined by Pratap and Bonga (1993). These authors observed changes to the ultrastructure of the gill epithelium indicated by the degeneration of pavement cells and chloride cells, and an acceleration in the turnover of the chloride cells after waterborne exposure. The effects of dietary Cd were similar, although delayed, relative to the effects observed with soluble Cd. Most other studies have similarly reported little to no impact on the fish consuming a diet containing Cd (Franklin *et al.* 2005; Dang & Wang 2009).

As opposed the dominant organic forms of arsenic and mercury (arsenobetaine and methyl mercury), inorganic metals such as Cd have a low potential to accumulate in fish muscle. Pratap and Bonga (1993) found that in contrast to arsenic and mercury, that Cd tended to accumulate not in the muscle, but rather in the viscera. The order of Cd accumulation strongly reflects the exposure pathway, with the gut > kidney > liver > gill > carcass > bone (for dietary Cd) (Pratap & Bonga 1993; Franklin *et al.* 2005; Dang & Wang 2009). On a whole-body basis, the net retention of Cd from the diet was < 1%, indicating that the gut wall forms an important protective barrier in reducing overall Cd accumulation into internal tissues (Franklin *et al.* 2005). Lundebye *et al.* (1999) observed similar tendencies in Cd accumulation, though the order was liver > intestine > gills in the fish fed in that study. In the study by Dang and Wang (2009), the fish marine grunts (*Terapon jarbua*) were either fed a Cd-contaminated diet or exposed to waterborne Cd for 4 weeks. It was found that Cd accumulated in different fish tissues (digestive tracts, gills or livers) in different extents. Accumulation was greatest in the liver (5.0–6.3 μg/g), followed by the digestive tract (0.8–3.2 μg/g) and gills (0.3–2.7 μg/g). Additionally, retention of Cd was greater from the diet than from a waterborne source (Franklin *et al.* 2005).

Interactions are also known to occur between Cd and Ca in terms of the effects on fish (Pratap & Bonga 1993). These authors observed that a high calcium concentration in the water reduced the impact of waterborne Cd but had no effect on the impact of dietary Cd on *Oreochromis mossambicus*. The addition of Ca^{2+} to diets of rainbow trout (*Oncorhynchus mykiss*) was observed to reduce on a short-term basis the whole-body uptake of both waterborne Ca^{2+} and Cd by > 50%. This consequently resulted in a much lower chronic accumulation of Cd (via either the water or diet) into the fish's body tissues. Based on these observations it was suggested that Ca^{2+} and Cd share common pathways and transport mechanisms across the gill and gut. It was postulated that increased gastrointestinal Ca^{2+} uptake caused a downregulation of both branchial and gastrointestinal Ca^{2+} uptake and therefore also influenced Cd uptake (Franklin *et al.* 2005). Atlantic salmon exposed to dietary Cd showed a disturbance of Ca homeostasis and depletion of Ca stores (Berntssen *et al.* 2003).

Another metabolic response often observed in fish in their response to the presence of dietary Cd has been the induction of the expression of the metal binding protein metallothionein (MT). However, it is important to note that the induction of MT is not Cd specific but has a regulating role for several essential and non-essential metals. In the study by Dang and Wang (2009), with the fish *Terapon jarbua*, the expression of MT was induced in response to Cd accumulation but returned to the control levels after an extended exposure period. The exception for this was the hepatic MT induction resulting from either waterborne or low dietary Cd exposure. The presence of Cd in the metallothionein-like protein (MTLP) fraction increased over exposure time, and it accounted for almost 60% of the Cd in the livers and 80% Cd in their digestive tracts by the end of the exposure period. Dang *et al.* (2001) also studied the effects of dietary Cd on MT and cortisol receptor (GR) immunoreactivity in the branchial epithelium of the Atlantic salmon (*S. salar*). In that study, Cd was fed at different concentrations (0.2, 5 and 125 mg/kg). Calcium, sodium, chloride and cortisol levels in the plasma were not affected after an 8-week study. Notably, in that study, Cd accumulation and a marked stimulation of MT expression were seen only in the chloride cells in the gills of fish fed the highest Cd dose. The authors concluded that the Cd entering the intestine also entered the gills, where it accumulated in the chloride cells and stimulated MT expression. (Dang *et al.* 2001). Berntssen *et al.* (2001) exposed Atlantic salmon to increased levels of dietary Cd. The highest increase in MT levels was found in the kidney, and MT levels increased disproportionally to Cd accumulation. It was concluded that MT was not directly associated with long-term Cd accumulation.

The EFSA established a TWI of 2.5 μg/kg bw for Cd in 2009. In 2010, the JECFA reviewed its previous evaluation on Cd and established a provisional tolerable monthly intake (PTMI) of 25 μg/kg bw, which corresponds to a weekly intake of 5.8 μg/kg bw. Based on the as low as reasonably achievable (ALARA) principle, the EU has established a maximum limit for Cd of 50 μg Cd/kg in fillets of most fish species. For a list of species, including eel and mackerel, the limit has been raised to 100 μg Cd/kg, for bullet tuna the limit is 200 μg Cd/kg, and for swordfish and anchovy it is 300 μg Cd/kg.

Lead

Lead (Pb; atomic number 82, atomic weight 207.2) is considered one of the most toxic non-radioactive metals. Ingestion of any measurable amount can have negative health effects on animals (Davis *et al.* 1990; Humphreys 1991). When ingested, Pb damages the nervous system and causes a range of neural disorders. Excessive Pb also causes blood disorders in mammals. Lead is considered a neurotoxin that accumulates in both soft tissues and bones (Davis *et al.* 1990; Humphreys 1991). In early studies that evaluated the uptake of Pb by rainbow trout from both waterborne and dietary sources, it was found that Pb added to the diet was poorly available to fish. However, when solubilized a 21-day LC_{50} of 2.4 mg/L was observed. It was apparent that the dietary Pb used was poorly absorbed and that most of the Pb consumed was subsequently found in the faeces (Hodson *et al.* 1978). In a more recent study with rainbow trout, the accumulation of Pb(II) from three diets with different levels of lead nitrate ($Pb(NO_3)_2$; 7, 77, and 520 mg/kg) and a Pb-free control diet (0.06 mg/kg) were fed to for a 21-day period (Alves *et al.* 2006). Over the course of the study, the accumulation of Pb was determined in various tissues (gills, liver, kidney, intestine, and whole carcass), red blood cells (RBC) and plasma. The accumulation of Pb from the diet occurred in a dose-dependent manner in all tissues except plasma. The intestine had the greatest Pb concentration (17.8 μg Pb/g tissue wet weight), although high concentrations were also observed in the kidney (2.4 μg Pb/g tissue wet weight) and liver (1.9 μg Pb/g). For each dietary treatment, the highest concentrations were observed by day 21. In the blood, it was noted that the RBCs accumulated the most Pb (1.5 μg Pb/g) when compared to the plasma (0.012 μg Pb/g). The efficiency (gain/intake) of Pb retained in the fish decreased with increasing dietary Pb concentrations/intake. Growth, survival, plasma protein and haematocrits of the fish were not significantly affected by dietary Pb intake. The levels of plasma Ca^{2+} decreased early after the commencement of the experiment, whereas Mg^{2+} levels decreased during the middle of the experiment in both the 77 and 520 mg/kg dietary treatments. However, both the Ca^{2+} and Mg^{2+} levels in the plasma stabilized by day 21 of the study. Neither of the ionoregulatory parameters of branchial Ca^{2+} and Na^{+} influx rates were affected by dietary Pb, except early in the study when Na^{+} influx rates were elevated. The results also showed that the intestine was a site of chronic toxicity of Pb from the diet.

In a study on tilapia (*Oreochromis niloticus*), fish were fed diets containing 0, 100, 400, or 800 mg/kg of Pb(II) as $Pb(NO_3)_2$ (Dai *et al.* 2012). At the end of the 60-day study, the fish were sampled to analyse the effects of dietary Pb intake on accumulation in different tissues. It was observed that Pb accumulation in tissues increased in line with increasing dietary Pb concentrations. Notably, the Pb accumulated the following tissues in order of concentration: posterior kidney>bone>liver>gill>spleen>testis>muscle>brain. The increase in dietary Pb level also correlated with a decline in glutathione content, GSH-Px and SOD activities. Some dose-dependent DNA damage in peripheral blood cells was also observed.

Mercury

Mercury (Hg; atomic number 80, atomic weight 200.6) and most of its compounds are renowned for being extremely toxic. Mercury can be biomethylated (usually as a product of microbial methylation) to form organomercury (organic) compounds such as methylmercury (meHg), which is more correctly referred to as 'monomethylmercury(II) cation'. These organic forms of the metal are the most toxic (Bidstrup 1964; Valle and Ulmer, 1972; Clarkson 1997). The chemical form of methylmercury in fish has been identified as methylmercury-cysteine (CH_3Hg-cyst), and is probably part of proteins (Harris *et al.* 2003). The acute toxicity of the pure chemical methylmercury forms (such as CH_3HgCl) is higher than the methylmercurycysteine form (Oyama *et al.* 2000; Harris *et al.* 2003).

Methylmercury is often formed in aquatic systems, and as such it tends to be accumulated from bacteria through to piscivorous fish via aquatic food chains (Harris *et al.* 2003). Because of this close association with aquatic environments, Hg is also the best studied of the heavy metals in terms of its impact on fish and their role in the food chain. Notably, species of fish that are in a high trophic position in the food chain, such as sharks and scrombrids (mackerels and tunas), tend to contain higher concentrations of meHg than other species, as it continues to accumulate in each animal upon consumption, a process referred to as biomagnification (Rivers *et al.* 1972; Renzoni *et al.* 1998). Thus, species that are of a high trophic level can amass body burdens of meHg that can be orders of magnitude higher than the species they consume (Rivers *et al.* 1972). Due to this biomagnification process, fish and other aquatic species are considered as one of the main sources of meHg exposure in the human diet (Rivers *et al.* 1972; Renzoni *et al.* 1998). However, the concentration of meHg in a fish depends on a range of factors, including the species, age and size of the fish and the water body the fish came from as there are distinct geographic differences across the world (Driscoll *et al.* 2013). Notably, those fish that inhabit more acidic water bodies tend to have higher levels of meHg (Fitzgerald & Clarkson 1991). In aquaculture terms, attention has been given to the sources of fishmeals used. In a survey of a sample of Norwegian salmon feeds, Berntssen *et al.* (2010a) found that meHg was the dominant form present, comprising more than 80% of the mercury content.

Mercury has been known to be toxic to humans, but it was with the outbreak of the so called Minamata disease where fishermen and their families were exposed to high levels of meHg that showed the tragic effects of marine mercury pollution. Methylmercury is especially toxic to the nervous system (Eto *et al.* 2010) and may cause neurological damage at the population level if the intake is high (Grandjean *et al.* 1995). The effects of mercury have been reported to cause both chronic and acute effects to animals ingesting it (Bidstrup 1964). No impact on either survival or growth was observed in any of the treatments in a detailed study by Berntssen *et al.* (2003); however, brain oxidative stress and pathological alterations were observed. In that study, juvenile (parr) Atlantic salmon (*S. salar* L.) were fed on diets supplemented with either mercuric chloride (0, 10 or 100 mg Hg/kg) or methylmercury chloride (0, 5 or 10 mg Hg/kg) to assess the effects of inorganic and organic dietary mercury on lipid peroxidation and neurotoxicity in the brain. There was significant accumulation of meHg in the brain of the fish fed 5 or 10 mg/kg diets, whereas the inorganic mercury was only significantly elevated in the brain only from fish fed the 100 mg/kg diet. In another study, Berntssen *et al.* (2004) reported that tissue MT induction and intestinal cell proliferation appeared to be useful and quantifiable early indicators of toxic mercury exposures. Based on the absence of induction of these early biological markers such as MT and cell proliferation, no-observed-effect-level (NOELs) could be set to 0.5 mg/kg for dietary methylmercury and 1 mg/kg for inorganic mercury. Lowest observed effect levels (LOELs) levels could be set to 5 mg/kg for meHg and 10 mg/kg for inorganic mercury.

The short-term absorption, distribution and elimination dynamics of meHg in fish were examined in the white sturgeon (*Acipenser transmontanus*) over a 48-h exposure by Huang *et al.* (2012). Diets containing meHg chloride were intubated into the fish at a range of doses (0, 250, 500 or 1000 μg Hg/kg body weight). Following intubation, repeated sampling of the blood and urine from the fish occurred over the following 48-h period, after which the fish were euthanized to measure Hg tissue concentration and distribution. The uptake of Hg into the blood of the fish peaked within 12 h, and remained elevated for nearly 48 h. There was a clear relationship between blood Hg concentration peaks and the dietary dose groups. Changes in blood Hg profiles could be described by a first-order rate kinetic function in all the treatments, suggesting that transfer was passive and not facilitated. The Hg concentration asymptote and rate of absorption (K) were observed to be dose dependent, with K-values ranging from 0.27 to 0.62 at the highest dietary meHg level. Digestibility of the meHg was highest (92–100%) at the lowest dietary dose and declined with increasing dietary content. Chou (2007) implicated that high dietary sodium and calcium levels fed to salmon during the parr–smolt adaptation period contributed to the elevation of Hg in their kidney and gills. Otherwise though, Hg accumulation dynamics over the grow-out period were observed to increase in a linear relationship to Hg uptake.

The longer term absorption dynamics and turnover of meHg from feed by the Atlantic cod (*Gadus morhua* L.) were examined by Amlund *et al.* (2007). After an initial lag period of 10 days, a continuous accumulation of meHg was observed in the muscle tissue over a three-month exposure period to meHg (0.95 μg Hg/g feed). At the end of this period, the concentration in the muscle tissue was 0.38 μg Hg/g wet-basis, and the meHg comprised 90–95% of the mercury present. Following the 3-month uptake period, the turnover dynamics were examined by feeding a low Hg diet. During this period, the reduction of meHg from muscle was slow with an estimated elimination half-life of 377 days.

Chou (2007) examined the distribution of the Hg in farm reared Atlantic salmon by measuring the concentrations of Hg in the muscle, liver, kidney and gill tissues of in response to the various dietary Hg concentrations. The greatest accumulation of Hg occurred in the order of kidney > gill = liver > muscle irrespective of dietary dose. The highest Hg peak concentrations were observed for both the kidney and gill during the parr–smolt period, while for the liver and muscle mercury peaked 1 month later after transfer to the grow-out site. A similar response was seen in the white sturgeon, by Huang *et al.* (2012), who observed that deposition of meHg was greatest in the gastrointestinal tract > kidney > spleen > gill > heart > liver > brain > white muscle and remaining whole body. At the end of the experiment, it was noted that the Hg was preferentially distributed to the most metabolically active tissues. Of the total meHg intake from a 1000 μg dose, >26% was recovered from the gastrointestinal tract, 14.7% from the muscle, 10.1% from the liver, 2.6% from the gills, 1.8% in the blood, 1.0% in the spleen, 1.0% in the kidney, 0.2% in the heart, 0.08% in the brain and 42.7% from the remaining whole body (although it was not clear what this included or excluded from the other organs). Only 0.06% was accounted for in the urine. Nøstbakken *et al.* (2012) reported contrasting results with the accumulation of meHg reported as being several folds higher in the kidney compared to other tissues. In that study, Atlantic salmon were fed elevated levels of dietary meHg for an 8-week period. During this period, the meHg-exposed fish accumulated significantly more Hg than control fish. Further analysis of the proteome revealed the differential abundance of 26 specific proteins in the kidney. The specific proteins identified indicated that meHg had affected metabolism, inflammation, oxidative stress, protein folding, and cell-structural components.

Amlund *et al.* (2007) found that the retention efficiency of meHg from feed was estimated at 38%. Of the meHg retained, more than 99% of it in the muscle was found in the protein fraction, where it was incorporated into larger peptides or proteins. Nøstbakken *et al.* (2012) also had observations consistent with these findings. In the study by Chou (2007), using farm reared Atlantic salmon, it was noted that the results showed dietary Hg loadings on marketable size salmon were within the tolerance limits of FDA and US EPA criteria. Additionally, it was noted that the rapid growth of salmon and comparatively low dietary Hg of manufactured feeds progressively reduced the uptake of Hg in farmed salmon.

The histopathology of samples of gills, olfactory epithelium, kidneys and liver were studied by de de Oliveira-Ribeiro *et al.* (2002) who examined the effects of a single dietary dose of inorganic Hg and meHg (0.26 and 0.05 μg·Hg/g body weight), when fed to the Arctic charr (*Salvelinus alpinus*). The distribution of the different forms of the dietary Hg in the intestinal epithelium was determined using ^{203}Hg as a tracer. The liver was largely unaffected by the intake of inorganic Hg, but intake of meHg had acute effects, with severe necrosis and alterations of cytoplasmic organization observed. Use of the ^{203}Hg tracer showed that meHg was found at very specific locations on the intestinal epithelial surface, whereas inorganic Hg was distributed evenly across the intestinal epithelium. A similar degree of cellular damage was also seen by Mela *et al.* (2014) in the trahira (*Hoplias malabaricus*) when fed elevated meHg levels. In this study, the liver showed leucocyte infiltration, an increased number of melanomacrophage centres (MMC), various necrotic areas and lesions. There was also an increase in the disorder of the cytoskeletal organization of the liver cells, suggesting a strong effect of meHg on organellar positioning and movement, vesicular traffic and secretion. The head kidney also showed large necrotic areas with an increased number of MMC, phagocytic areas and atypical cells. Cambier *et al.* (2009, 2012) studied the impact of meHg on mitochondria isolated from muscles of zebrafish fed meHg contaminated feed and found that the organelles presented clear structural abnormalities under electron microscopy observation. Cambier *et al.* (2012) used the transmission electron microscopic observation to confirm the impairment of the optical tectum in zebrafish (*Danio rerio*) fed elevated levels of meHg. These researchers noted a decrease the nuclei area in contaminated granular cells relative to those cells from fish fed the control diet. Additionally, there was a lower density of cells in the contaminated tissue relative to uncontaminated tissues. These authors suggested that this might have resulted in impaired vision in those fish contaminated with meHg and therefore a result in a poorer adaptability to their environment as a consequence.

In the study by Berntssen *et al.* (2003) with juvenile Atlantic salmon that observed no effects on either growth of survival, a suite of enzymatic parameters was also examined. Those fish fed the lowest level of meHg had a twofold increase in the activity of the antioxidant enzyme SOD in the brain. At higher levels of dietary meHg, a sevenfold increase was observed in lipid peroxidative products (thiobarbituric acid reactive substances, TBARS) and a subsequent decrease (1.5-fold) in antioxidant enzyme activity (SOD and GSH-Px). These fish also had clear pathological damage (vacuolation and necrosis) to their brain and reduced activity of a key neural enzyme (fivefold reduction in monoamine oxidase (MAO) activity). These enzymatic changes were concomitant with a reduction in post-feeding activity. Fish fed the highest level of inorganic Hg also had reduced neural MAO activity and pathological changes in the brain. However, despite these clear pathologies, the neural SOD and GSH-Px enzyme activities, lipid peroxidative products (TBARS), and post-feeding behaviour were not affected. Compared with other organs, the brain is considered to be particularly susceptible for dietary meHg induced lipid peroxidative damage. Interestingly, the lower levels of dietary meHg induced some protective redox defences in the brain based on the induction of the activity of the antioxidant enzyme SOD. However, at the higher levels of meHg intake, protective redox defences in the brain were overcome and there were indications of lipid peroxidative damage (elevated TBARS).

Cambier *et al*. (2009, 2012) studied the impact of meHg on mitochondrial structure and function, in the zebrafish. These researchers observed a strong inhibition of mitochondrial respiration and cytochrome c oxidase (COX) activity and proposed that this represented a defect at the level of ATP synthesis due to meHg. When they measured the rate of ATP release by myocytes using either pyruvate and malate or succinate as substrates, they found a reduction consistent with this notion.

The studies by Cambier *et al*. (2009, 2012) on the impact of meHg on mitochondrial function in the zebrafish found that there was a decoupling of mitochondrial oxidative phosphorylation in the muscle cells of zebrafish, but that the brain mitochondrial respiration was not affected by exposure to meHg. Supporting these observations was a sixfold increase in the expression of the gene encoding the succinate dehydrogenase Fe/S protein subunit. Additionally, there was also an upregulation of three genes encoding for calcium transporters, suggesting a perturbation of calcium homeostasis as a consequence of meHg intake. The upregulation of the glial fibrillary acidic protein and two glutathione *S*-transferase genes, along with a downregulation of a glutathione peroxidase gene support the notion of a meHg-induction of oxidative stress and inflammation. The molecular toxicity of meHg in the liver, brain and white muscle of Atlantic salmon was studied using a series of diets in a factorial design based on either fish oil (FO) or vegetable oil (VO) and enriched with or without 5 mg meHg/kg (Olsvik *et al*. 2011). As expected, the dietary oil type had clear effects on the fatty acid composition in the tissues, influencing the n-3 and n-6 status of various tissues. There was also clear differential accumulation of the meHg in the various fish organs examined, with the liver accumulating three times as much MeHg as the brain and white muscle. Effects of meHg on the transcription of haem oxygenase, tubulin alpha and Cpt1 genes in the liver were observed. There was some indication of interactive effects between meHg intake and dietary lipid type in all tissues. The authors went on to suggest that certain dietary fats may have some capacity to modulate effects of meHg toxicity in Atlantic salmon. In another study on Atlantic salmon fed elevated levels of dietary meHg a series of specific proteins were identified that indicated that meHg had effects on metabolism, inflammation, oxidative stress, protein folding and cell-structural components (Nøstbakken *et al*. 2012). Correspondingly, gene expression analysis also identified a few differentially regulated genes in the kidney and liver in the meHg fed fish compared to the control fed fish.

It has been suggested that elevated levels of vitamin C may offset some of the damage attributable to dietary meHg intake (Mozhdeganloo *et al*. 2015). A study undertaken to evaluate meHg-induced changes in liver enzymes and oxidative stress markers in rainbow trout (*Oncorhynchus mykiss*) explored the possible protective effect of vitamin C against these alterations. In an *in vitro* study, liver samples of fish were exposed to different doses of meHg (0.6, 1.2, and 2.4 μg/L) for 120 min. In an additional treatment, the liver samples were treated with vitamin C (17.2 μg/L) along with high dose (2.4 μg/L) of meHg. At each of the meHg doses, a significant increase in hepatic enzyme activities (alanine transaminase (ALT), aspartate transaminase (AST) and lactate dehydrogenase (LDH)) was observed. Additionally, the malondialdehyde (MDA) level, a marker of lipid peroxidation, was also elevated in each case. The addition of vitamin C to the highest meHg-exposed treatment led to a significant reduction in the MDA concentration and the hepatic enzyme activities and significant increase in levels of GSH and total antioxidant capacity. It was suggested that the addition of the vitamin C returned the values of the measured enzymatic parameters to levels that were comparable to those of the control group. Furthermore, selenium a essential mineral that is known to be relatively high in seafood, is known to interact with methylmercury and lower its accumulation in tissues (Dang & Wang 2011; Ordiano-Flores *et al*. 2012; Rasinger *et al*. 2017).

Radioactive metals and other radionuclides

Environmental and food/feed contamination with radioactive metals and other radionuclides was largely ignored until the advent of large-scale nuclear weapon proliferation in the 1960s and beyond (Garner & Comar 1972). Other non-military nuclear events, such as the Chernobyl disaster of 1986 and the more recent Fukushima disaster in 2011 have served to reaffirm the high-risk associated with such contaminants in the environment and food-chain (Akiba 2012; Sato *et al*. 2013; Steinhauser *et al*. 2014). The key contaminants of concern released to the environment are radioactive isotopes such as caesium-137, iodine-131, strontium-90 and some other radionuclides (Hosoda *et al*. 2013). These radioactive isotopes are generated during the nuclear fission that occurs during the splitting of uranium into two smaller nuclei and the released energy used for heat generation. Most of these radionuclides have long half-lives; caesium-137: 30-years, strontium-90: 30-years, meaning that they are persistent in the environment for a long time after release. Other naturally occurring radioactive materials include polonium-210, radium-226 and radium-228 (De Bortoli & Gaglione 1972; Smith-Briggs & Bradley 1984). Human health effects from both anthropogenic and naturally occurring radionuclides are of concern due to their bioavailability and bioaccumulation characteristics in seafood used for human consumption. There is little aquaculture-based data available with respect to radionuclide contamination, with most data coming from ecological studies.

Fish are often used as bioindicators of aquatic pollution, including that of radionuclides. The genotoxic effect to common carp (*Cyprinus carpio*) of gamma radiation was assessed were the fish were irradiated with different doses (2, 4, 6, 8 and 10 Gy) of gamma rays using a teletherapy machine (Praveen *et al.* 2015). Following this irradiation, an assay was performed on nucleated erythrocytes from the fish after 24, 48 and 72 h of irradiation to assess the degree of DNA damage. Results from the assay demonstrated that a significant increase in DNA damage was observed at all the doses of gamma radiation relative to the unirradiated controls and that the degree of DNA damage occurred in a dose-dependent manner.

Abnormal DNA distributions have been observed in cells from several of the fish from Chernobyl sites relative to the control sites. The impact of chronic radionuclide exposure to populations of four species of fish; channel catfish (*Ictalurus punctatus*), crucian carp (*Carassius carassius*), carp (*Cyprinus carpio*) and tench (*Tinca tinca*), inhabiting contaminated sites near the Chernobyl Nuclear Power Plant (CNPP) were compared with control populations from two uncontaminated locations distant from the plant (Dallas *et al.* 1998). Flow cytometric analysis of whole blood as well as separate erythrocyte and leucocyte components, identified some key changes in those fish from the contaminated sites relative to the control sites. Perturbations in the cell cycle in fish from the Chernobyl sites were also detected, with a higher percentage of cells in G2/M phase relative to the controls. Leucocytes were more sensitive to radionuclide exposure than erythrocytes, presenting a greater number of abnormal DNA distributions.

The other major nuclear accident in recent times, the Fukushima accident in March 2011, allowed for the study of radionuclide contamination in ayu (*Plecoglossus altivelis*) (Tsuboi *et al.* 2015). Following the accident, fish were exposed to highly contaminated silt containing radiocaesium (Cs-134 and Cs-137). In assessing the impact on the fish, samples were analysed from the riverbed (algae and silt) and the internal organs and the muscle of Ayu from five river systems in the Fukushima Prefecture. The levels of radiocaesium in the environmental samples and the tissue of the fish were closely correlated and it was perceived that this was reflective of the fish's feeding habits. There was also a positive correlation in the radiocaesium levels in the muscle and the internal organs of the fish, although the levels in the muscle were considerably lower than those in the internal organs.

An experiment examining the chronic and acute exposure of fish to a similar total cumulative level of gamma radiation was reported by Anbumani and Mohankumar (2012). In this study, freshwater carp (*Catla catla*) were subjected to protracted (0.002 Gy/min) and acute (3.2 Gy/min) gamma radiation to a total dose of 5 Gy. Following each exposure, blood samples were collected at regular intervals over an extended period (from day 3–202). The blood was analysed using an erythrocyte micronucleus assay. A range of cellular anomalies were observed on erythrocyte nuclei including micronuclei, deformed nuclei, nuclear buds, nuclear bridges, vacuolated nuclei, binucleated cells and apoptotic cells. A range of cytoplasmic abnormalities were also detected, including vacuolated cytoplasm, anisochromasia, echinocytes and enucleated cells. Both chronic and acute exposure to gamma radiation caused a significant increase in nuclear and cytoplasmic abnormalities. However, the damage was significantly higher after acute exposure than chronic exposure.

The level of DNA damage (as indicated by the alkaline unwinding method) in a range of tissues (liver, gills and red blood cells) from largemouth bass (*Micropterus salmoides*) exposed to contamination by mercury and radiocaesium was examined by Sugg *et al.* (1995). An increased concentration of the toxicants was correlated to increased DNA damage. However, it was noted that the different tissues examined responded differently to the different contaminants. Notably, erythrocytes generally showed the greatest level of DNA damage, while the liver tissue was the least affected. Sugg *et al.* (1996) also examined the levels of contamination and genetic damage associated with radiocaesium in catfish (*Ictalurus punctatus)* from the CNPP cooling pond and a non-contaminated control site. Generally, fish obtained from the cooling pond exhibited greater genetic damage, primarily in the form of DNA strand breaks with a few micronuclei being observed in some of the contaminated fish. It was surmised that the amount of DNA damage was correlated to the concentration of radiocaesium in individual fish.

Few histological approaches in examining the impact of radionuclides on fish have been reported. One study examined the state of the reproductive system in several fish species exposed to radiation from the CNPP was reported (Belova *et al.* 2007). In this study, it was observed that the total number and the severity of histological disturbances in gonads of the various fish species studied were positively correlated with the levels of pollution in the different water bodies sampled for the study. Based on the number and type of histological anomalies, a species specificity in the response to the background radiation was identified and was suggested to be trophically linked.

A trophic cascade effect of the transmission of Cs-137 was reported by Rask *et al.* (2012). They observed that the earliest accumulation of Cs-137 was in the planktivorous species like crustacean zooplankton (1000 Bq/kg), before occurring in those species in higher trophic levels like perch (*Perca fluviatilis*: 13 600 Bq/kg) and for pike (*Esox lucius*: 20,700 Bq/kg). Notably, with the higher the trophic level the greater the accumulation.

The accumulation dynamics of a range of radionuclides (Ra-226, Th-232, Th-230 and Th-228) from water, sediment and prey items into the bone and muscle of white suckers (*Catostomus commersoni)* was studied across several lakes in Canada (Pyle & Clulow 1997). The authors noted that there was a correlation between environmental levels and the levels of these radionuclides in the fish tissues; however, the relationship was not a linear one but was better described by a power function.

Most studies across a range of marine and freshwater species have shown that the retention of Ra-226 and Ra-228 in bone is higher than that of muscle (Iyengar, 1984; Iyengar & Rao 1990; Neff 2002). Assessments of the retention of radium based on the whole organism approach have been demonstrated to overestimate the level of radium in the edible portion by uniformly apportioning the loading (Iyengar MAR 1984). It has been noted that a minimum of 40% of the total radium a fish is exposed to accumulates in the bones and only 6% ends up in the muscle (Neff 2002). It has been suggested that because radium is chemically similar to calcium that it is more likely to concentrate in bones, shells, and exoskeletons (Meinhold & Hamilton 1992).

A range of water chemistry parameters have been implicated in the accumulation dynamics of Cs-137 in fish (Smith *et al.* 2009). Because of role that potassium plays in mineral balance and uptake in fish it was suggested that water potassium levels might influence Cs-137 uptake. An analysis of over 1000 samples from nine European lakes following the Chernobyl accident identified that accumulation of Cs-137 in fish was negatively correlated with lake potassium levels. These authors also found a strong effect of fish size on accumulation.

Generally, the risk to humans of eating fish contaminated with radionuclides is considered to be very low, even following nuclear accidents like the ones of Chernobyl and Fukushima. The study examined the links between radioactivity and possible health impairments to humans, and also examined the doses attributable to the Fukushima-derived radionuclides and the naturally occurring radionuclides present in the environment (Fisher *et al.* 2013). That study determined that levels of radionuclides in both the marine biota and human fish consumers were dominated by the naturally occurring alpha-emitter Po-210 and that the Fukushima-derived doses were three to four orders of magnitude below the naturally occurring Po-210 derived doses. Those doses that the marine biota was exposed to were about two orders of magnitude below the protection level proposed for ecosystems (10 μGy/h). The authors argued that such doses are no worse that the doses that people are routinely exposed to from other naturally occurring radionuclides in many food items, medical treatments, air travel or other background sources.

Mycotoxins

Mycotoxins are secondary metabolites produced by filamentous fungi that can cause toxic responses in a variety of animal species (Nácher-Mestre *et al.* 2015; Anater *et al.* 2016; Binder 2007; Matejova *et al.* 2017). They all have a significant impact on performance in animal production by inducing acute and/or long-term chronic effects that result in a range of toxic effects including carcinogenic, oestrogenic and teratogenic or other toxic effects (Anater *et al.* 2016; Jouany 2007). However, the extent of the toxicity can be quite specific to both the type of toxin and the species of animal which consumes it (Binder *et al.* 2007; Williams & Blaney 1994).

There are a variety of mycotoxins produced by different fungal contaminants. Each of these mycotoxins falls within one of six major groups: aflatoxins, ochratoxins, citrinin, ergot alklaoids, patulin and fusarium toxins (Table 2). Within each group, there are different toxins, some are basic variants on a generalized structure, while others can be quite diverse. Each of these toxin groups is derived from fungal contamination, usually of grain or legumes (Anater *et al.* 2016).

Studies on the toxicity of aflatoxin B_1 to rainbow trout, Coho salmon and channel catfish were reported by Halver (1965). In these studies, the survival of fish fed diets with varying concentrations of aflatoxin B_1 was assessed over various periods of time. Trout were fed diets with 0.15, 0.5 or 1.0 mg/kg of aflatoxin B_1. Within a 5-day period, there was a 100% mortality of fish that were fed the diet with 1.0 mg/kg. Over the same period, only half the fish fed the 0.15 mg/kg died. Half those fish that were fed the 0.5 mg/kg diet died within the first day. A similar response was seen with Coho salmon, where fish fed the 1.0 mg/kg di *et al* l died within 10 days. By increasing the aflatoxin B_1 concentration in the diet to 3 mg/kg, 90% of the fish died within 5 days. Channel catfish were more resilient with only 40% of the fish dying when fed 3 mg/kg aflatoxin B_1 over a 5-day period, with 20% of the catfish dying within 1 day when fed the 1.0 mg/kg dose. Increasing the aflatoxin B_1 dose to 5.0 mg/kg did not increase the losses observed within 1 day; however, a threefold increase to 15.0 mg/kg increased the mortality to 80% over the same period. These results demonstrated that there are widely variant tolerances among different fish species. Atlantic salmon fed 2 or 6 mg/kg pure deoxynivalenol (DON), 0.8 or 2.4 mg/kg pure ochratoxin A (OTA), or no added toxins, for up to 8 weeks. A different effect pattern was found for the two toxins within the dosage levels tested. For DON, the results indicate that the maximum recommended levels of DON in the current legislation on animal feed in the EU at 5 mg/kg feed (European Commission, 2006a) is inappropriate and not suited for salmon and most other examined fish species. Also, the Norwegian maximum level of DON in feed for fish at 2 mg/kg (Bernhoft *et al.* 2017) does not appear to be sufficient to protect juvenile Atlantic salmon from adverse effects. For OTA, a low sensitivity for adverse effects seems to be in accordance with the finding of rapid elimination and possibly induced elimination mechanisms of OTA in the salmon (Bernhof *et al.* 2017). This is different from the sensitivity and dose–response effects shown in channel catfish fed OTA. The actual maximum recommended level for OTA in fish feed in the EU at 0.25 mg/kg (European Commission, 2006b) does protect the Atlantic salmon from adverse effects (Bernhoft *et al.* 2018).

It has been argued that the most appropriate control point to limit the impact of mycotoxins is during crop production, where proper crop rotation and appropriate fungicide administration can limit the development of fungal infections of the crops. The use of in-feed treatments or adsorbents has also been advocated (Binder 2007; Jouany 2007). Certain adsorptive compounds have been used for a general reduction in the potency of mycotoxins (Jouany 2007; Zhu *et al.* 2016). Additionally, alternative strategies such as the use of enzymatic or microbial detoxification, have also been used for counteracting impacts of certain fungal toxins (Binder 2007; Zhu *et al.* 2016). The compounds are usually heat stable and therefore are not impacted by pasteurization or denaturation (Anater *et al.* 2016).

The incidence of carry-over of mycotoxins into animal products from contaminated feed was reviewed by (Kan & Meijer 2007). It was reported that up to 6% of the aflatoxin from feed found its way into the milk of dairy cows. In contrast, the carry-over of DON, zearalenone (ZON) and fumonisin to any animal products appears negligible. Ochratoxin A accumulates in the kidney, liver and blood of animals that consume it. Nácher-Mestre *et al.* (2015) investigated the levels of mycotoxin in plant-based Atlantic salmon and gilthead seabream. In addition to eight mycotoxin under EU regulation/guidance in feed and feed ingredients (AFB1, DON, ZEN, OTA, FB1 + FB2, T-2 and HT-2), 10 additional mycotoxins of potential relevance for food safety are included (AFB2, AFG1, AFG2, FB3, nivalenol (NIV), 3-acetyldeoxynivalenol (3-AcDON), 15-acetyldeoxynivalenol (15-AcDON), diacetoxyscirpenol (DIA), fusarenon-X (Fus X) and neosolaniol (NEO)) were included in this study. The feed mycotoxins levels reflected the feed ingredient composition and the level of contaminant in each feed ingredient. In all cases, the studied ingredients and feeds showed levels of mycotoxins below MRL established by the Commission Recommendation 2006/576/EC. No mycotoxin carry-over was found from feeds to edible fillets of salmonids and a typically marine fish, such as gilthead sea bream. In a study with Atlantic salmon fed diets fortified with pure DON and OTA concluded that the risk to human health from the consumption of salmon-fed diets containing maximum recommended levels of these toxins to be negligible (Bernhof *et al.* 2017).

Increasing levels of globalization in the trade of agricultural commodities has increased the awareness of mycotoxins as differing levels in grain production are evident among different countries and the biosecurity risks these present (Binder *et al.* 2007). Additionally, this awareness in mycotoxin issues in food and feed production has also risen since analytical methods have become noticeably more sophisticated and more available at all points of the supply chain. The study by Binder *et al.* (2007) examined the distribution and severity of mycotoxin contamination across Europe and the Asia-Pacific found that more than half of the materials sampled in Europe were contaminated at levels above the limit of quantification of the methods applied, while one-third of the Asian-Pacific sourced samples were positive. European samples had DON, ZON and trichothecene (T-2) toxin as the major contaminants, whereas those materials from the Asia-Pacific tended to be contaminated with DON, ZON, fumonisins and aflatoxins.

Antimicrobial residues

The residues of antimicrobials have raised concerns in the food chain for a variety of reasons and the level of legislative intervention in their management varies worldwide (Barton 1998; Bruno 1989; Castanon 2007; Reig & Toldrá 2008). The control of the presence of antimicrobials in animal foods and feeds has been particularly tightly regulated in the EU through Directive 96/23/EC, which mandates the monitoring of certain substances and residues in live animals and animal products for use in the human food chain. A crucial part of the monitoring in this directive is the independent screening of veterinary drug residues in live animals, feeds and animal products (Berntssen *et al.* 2014). For each antimicrobial, critical withdrawal times have been stipulated and the presence of maximum residue levels for each tissue type or product (MRL) for each are defined.

The principal concern about antimicrobial residues has been based on the development of antimicrobial resistance

Table 2 Groups and varieties of mycotoxins and their origins and examples of limits

Group	Mycotoxin	Fungal origin	Crop origin	Toxicity basis	Limits
Aflatoxins	–Aflatoxin B_1 –Aflatoxin B_2 –Aflatoxin G_1 –Aflatoxin G_2 –Aflatoxin M_1 –Aflatoxin M_2 –Aflotoxicol –Aflatoxin Q_1	*Aspergillus* sp.	–Peanuts –Corn –Cottonseed	–Hepatotoxin –Carcinogen –Teratogen –Immune suppression	Aflatoxin B1 – rainbow trout $LD_{50} < 0.15$ mg/kg – coho salmon $LD_{50} < 0.15$ mg/kg – channel catfish $LD_{50} < 5.0$ mg/kg
Ochratoxins	–Ochratoxin A –Ochratoxin B –Ochratoxin C	*Aspergillus* sp. *Penicillium* sp.	–Cereals –Coffee –Fruit	–Carcinogen –Nephrotoxin	Ochratoxin A – European seabass $LC_{50} < 0.28$ mg/kg BW – channel catfish NOEL < 0.5 mg/kg
Citrinin	Citrinin	*Aspergillus* sp. *Penicillium* sp.	–Wheat –Rice –Corn –Barley –Oats –Soy products	–Nephrotoxin	No dose response data identified
Ergot alkaloids	Ergotamine -6,8-dimethyl-ergoline derivatives -Lysergic acid derivatives	*Claviceps* sp.	–Wheat –Rye –Barley –Corn –Sorghum	–Neurotoxin –Vasoconstrictor	No dose–response data identified
Patulin	Patulin	*Aspergillus* sp. *Penicillium* sp. *Paecilomyces* sp.	–Fruits –Vegetables	–Genotoxin	No dose–response data identified
Fusarium	–Fumonisins –Trichothecenes –Deoxynivalenol –Zearalenone –Beauvercin –Enniatin –Butenolide –Equisetin –Fusarins	*Fusarium* sp.	–Wheat –Corn –Rice –Barley –Oats –Sorghum	–Hepatotoxin –Nephrotoxin –Oestrogenic –Protein synthesis inhibitor	Fumonisins – tilapia NOEL < 150 mg/kg Deoxynivalenol –rainbow trout NOEL < 0.3 mg/kg – Atlantic salmon NOEL < 3.7 mg/kg

LD, lethal dose; LC, lethal concentration; NOEL, no observable effect limit. References used: Manning *et al*. (2003); El-Sayed *et al*. (2009); Hooft *et al*. (2011); Döll *et al*. (2010); Gonçalves *et al*. (2018).

in bacteria, thereby limiting effectiveness of each antimicrobial as a means of subsequent human medical treatment (Barton 1998; Wegener *et al*. 1999). Over recent decades, multiresistant pneumococcal, glycopeptide-resistant enterococci and Gram-negative bacteria with extended-spectrum lactamases have become widely distributed across the world and are now a serious therapeutic problem to human medicine (Lee *et al*. 2001). Another concern for the presence of antimicrobial residues in the human food chain is the potential for allergic reactions (Lee *et al*. 2001). Some antimicrobials, such as penicillins have been reported to induce allergic reactions with reactions occasionally occurring in response to small amounts of the compounds in the food chain. It is acknowledged that misuse of antimicrobials in human medicine is the principal cause of this problem (Barton 2000). However, the once widespread use of antimicrobials in the animal feed sector has been implicated as a contributory factor (Lee *et al*. 2001). Although antimicrobials remain an important option in the treatment of sick animals, the once routine application of these chemicals in the animal diets to prevent infections and act as growth promoters is considered an abuse of their merits (Castanon 2007; Reig & Toldrá 2008).

As aquaculture has grown, a range of bacterial diseases have occurred that have caused both major production and animal welfare problems (Alderman & Hastings 1998). These diseases were originally almost exclusively controlled through the use of antimicrobial agents. More recently, in some aquaculture industries, the use of an effective range of vaccines has largely supplanted the use of many antimicrobials (Anderson 1992; Press & Lillehaug 1995). Within salmonid aquaculture, the use of antimicrobial agents for most bacterial diseases is now largely confined to emergency use in the event of failure of vaccine protection (Alderman & Hastings 1998). In addition to the increasing availability of vaccines, aquaculture is steadily developing a range of improved husbandry methods to reduce the impact of disease on the farmed fish population. Although there is evidence that antimicrobial resistance can be selected for in normal therapeutic use in aquaculture, the risks of transfer of such resistance to human consumers by any of the possible routes appears to be low (Alderman & Hastings 1998; Berntssen *et al*. 2014).

The risks that antimicrobials present within feed raw materials to aquaculture stems primarily from the residual levels of antimicrobials used in either poultry or mammalian meat production and the meals subsequently being rendered for use by the aquaculture sector (Berntssen *et al*. 2014; Bruno 1989). However, for most compounds, there is a withdrawal period prior to slaughter in either poultry or mammalian meat production that should minimize the potential of inadvertent transfer of antimicrobials to aquaculture species and subsequently humans (Codex Alimentarius Commission, 2017). While the withdrawal period is a good guideline, depending on the raw material, the MRL is more informative as specific meals may bioaccumulate compounds differently as was recently recognized with feather meal (Love *et al*. 2012).

The MRL reflect the maximum acceptable amount of a chemical that can residually remain in a specific tissue or product when that chemical is used based on its specified application. Samples with residues exceeding the MRL are generally those for which a prescribed chemical has not been applied or managed within its required practice (Jeong *et al*. 2010).

Antinutritional factors

While the nutrient composition of plant meals is often the positive selling point of these raw materials, it is clearly the ANF content that is their major 'Achilles heel'. Antinutrients, also referred to as biologically active substances, are essentially an evolutionary development of a chemical defence strategy by plants to provide some level of protection against being eaten. In this sense, many antinutrients are essentially a chemical defence mechanism being employed by plants. However, the variety of antinutrients found in the different plant species, l *et al* one their seeds, varies quite widely, both in diversity of antinutrient type and relative concentration (Table 3).

Since the seminal review by Francis *et al*. (2001a) on this topic there has been a consistent level of development of our understanding of the influences of the key ANF on fish. However, the assessment is far from comprehensive and in many instances, there is also an absence of data from multiple species with respect to influence of each ANF and/or thresholds to their effects. However, perhaps the most logical way to examine this knowledge set is by reviewing each of the key ANF's and considering how each affects the nutritional responses by fish and what potential mitigation strategies there are for each.

Alkaloids

Alkaloids are generally bicyclic, tricyclic or tetracyclic derivatives of the molecule quinolizidine (Petterson 2000). Data on the influence of alkaloids on fish and shrimp shows that alkaloids are generally considered a feeding deterrent because of their bitter taste (Glencross *et al*. 2006; Serrano *et al*. 2011, 2012; Smith *et al*. 2007). While the alkaloids are found primarily in the legumacae family (peas and beans), high levels are notably found in some varieties of lupins (Glencross *et al*. 2006). Present levels of alkaloids in most commercial lupin varieties of the species *Lupinus angustifolius* are usually less than 200 mg/kg. However, some varieties of *Lupinus luteus* (e.g. cv. Teo) have been released with alkaloids levels as high as 4000 mg/kg (Glencross *et al*. 2006). Moreover, wild-type varieties still found in their countries of origin may contain from 5000 to 40 000 mg/kg of alkaloids (Petterson, 2000).

In a study with rainbow trout, a threshold to alkaloid inclusion of ≤ 100 mg/kg of diet was reported for the alkaloid gramine by Glencross *et al*. (2006). This was reaffirmed in a series of more recent studies by Serrano *et al*. (2011, 2012) with the alkaloids lupinine and sparteine. Therefore, to avoid impacts of alkaloids certain thresholds can be observed with respect to the inclusion of potential sources of raw materials. Alkaloids are highly water soluble and therefore can be removed by water extraction methods (Petterson 2000).

Glucosinolates

Glucosinolates are a natural class of organic compounds largely found in the Brassicales order of plants as secondary metabolites (Brown & Morra 1997). They are water-soluble derivatives of glucose and amino acids, which also contain a sulfur molecule and a variable 'side-group'. There are about 130 different forms of glucosinolates, with each having a different side group, and it is this variation in the side group that is responsible for the variation in the biological activities of these compounds (Brown & Morra 1997). Some glucosinolates include sinigrin, which is the precursor to allyl isothiocyanate, glucotropaeolin which is the precursor to benzyl isothiocyanate, gluconasturtiin is the precursor to phenethyl isothiocyanate, and glucoraphanin is the precursor to sulforaphane (Brown & Morra 1997; Tripathi & Mishra 2007).

Table 3 A summary of key antinutritional factor (ANF) content of feed grains. Where data were available values are ranges of levels of each ANF found in each grain expressed as g/kg unless otherwise detailed

Common/Species name	Processing State	Alkaloids	Glucosinolates (mmol/kg)	Lectins (Units/mg)	Phytate	Protease inhibitors (UTI/g)	Oligosaccharides	Lignin	Saponins	Tannins	Polyphenolics
Barley/*Hordeum vulgare*	Whole/Raw	–	–	–	2.0–10.8	–	–	–	–	0.14–0.23	–
Camelina/*Camelina sativa*	Whole/Raw	–	16.3–40.3	–	12.0–32.3	–	–	–	–	1.50–4.39	–
Chick Pea/*Cicer arietinum*	Whole/Raw	–	–	ND	3.9–12.1	0.2–44.8	26–44	16.5–21.5	3.6–4.6	–	3.0–10.8
Corn/*Zea mays*	Whole/Raw	–	–	–	4.73–9.87	3.65	–	–	–	6.73	5.33
Cottonseed/*Gossypium hirsutum*	Solvent extr	–	–	ND	44.3	0	–	–	–	–	–
Faba Beans/*Vicia faba*	Whole/Raw	–	–	–	1.2–9.8	<0.2–12.8	34–54	2.0–5.0	13.3–39.0	0–15.0	0.03–0.37
Guar/*Cyamopsis tetragonoloba*	Whole/Raw	–	–	–	29.8	1.8	–	–	27.5	5.9	
Lupin/*Lupinus angustifolius*	Dehulled	0.02	–	ND	5	6	89	2–22	0.573	0	3
Lupin/*Lupinus albus*	Dehulled	0.23	–	ND	4	6	78	7	ND	1	15
Lupin/*Lupinus luteus*	Dehulled	0.03–4.09	–	ND	7	3	102	7	ND	0	2
Mustard/*Brassica juncea*	Whole/Raw	–	57–204	–	–	–	–	–	–	–	–
Oats/*Avena sativa*	Whole/Raw	–	–	–	7.9–10.1	–	–	–	–	–	–
Peanut/*Arachis hypogaea*	Dehulled	–	–	0.6–5.1	17.8–20.1	0.3–1.3	10–11	–	–	–	–
Peas/*Pisum sativum*	Whole/Raw	–	–	5.1–6.2	5.7–13.3	<0.2–11.1	24–66	0–3	0.7–1.9	0.07–7.50	0.09–0.92
Physic Nut/*Jatropha curcas*	Whole/Raw	–	ND	0.35–1.46	85.4–92.7	26.5–36.0	–	–	21.4–34.0	0.2	1.5–2.4
Rapeseed/*Brassica napus*	Solvent extr.	0.02	1.1–35.4	–	12.0–36.6	1.7–9.0	9.5–12.0	–	–	2.0–9.0	15
Sorghum/*Sorghum bicolor*	Whole/Raw	–	–	–	2.4–3.2	–	–	–	–	0.3–19	–
Soybean/*Glycine max*	Solvent extr	0.04	–	ND	9.0–19.5	1.8–10.0	55–99	–	49	0	5
Soybean/*Glycine max*	Cooked	–	–	ND	20.1	0.65	–	–	–	–	–
Soybean/*Glycine max*	Whole/Raw	0.29	–	5–20	8–20.1	0.2–86.1	40–88	–	56	8	5
Wheat/*Triticum aestivum*	Whole/Raw	–	–	–	3.0–13.9	21.7–46.3	–	–	–	0.9–2.0	1

Solvent extr: Solvent extracted. ND: not detected. A space (–) denotes that no data was found for this ANF for this feed grain. Data derived from: Rouanet *et al*. (1989); Attia *et al*. (1994); Petterson *et al*. (1999); Ejigui *et al*. (2005); Tripathi and Mishra (2007); Glencross (2008); Jezierny *et al*. (2010); Makkar et al. (2011); Russo and Reggiani (2012); Singh *et al*. (2012); Hixson *et al*. (2016).

In their own right, glucosinolates actually have little biological activity but depend on the enzymatic activation of myrosinase in the presence of water to cleave off the glucose group to produce the breakdown products of isothiocyanates, nitriles, thiocyanate anions and vinyloxazolidinethiones (Tripathi & Mishra 2007). Each of these glucosinolate breakdown products has some goitrogenic activity, which means they are substances that suppress the function of the thyroid gland by interfering with iodine uptake (Tripathi & Mishra 2007). These compounds induce hypothyroidism in most vertebrate animals and lead to reduced levels of the thyroid hormones triiodothyronine (T3) and thyroxine (T4). The modes of action of these bioactive compounds is closely involved with the synthesis of T3 and T4, notably thiocyanate anions compete with the active transport of iodine to the thyroid while vinyloxazolidinethiones blocks the coupling of sub-units of the precursors to T4. The consequence of hypothyroidic response in fish is usually manifested by a reduced metabolic rate leading lethargy, low appetite and subsequently poor growth (Burel *et al.* 1998, 2001).

Lectins

Lectins, also known as haemagglutinins, are proteins that possess as specific affinity for carbohydrate moieties (Peumans & Van Damme 1995; Sharon & Lis 1990). These proteins can cause the agglutination of erythrocytes, hence their alternative name. The primary antinutritional mode of action of lectins is their ability to reduce the absorption of nutrients from the gastrointestinal tract, resulting in intestinal hypertrophy, hyperplasia, pancreatic enlargement, increased liver weight, thymus atrophy and loss of muscle mass (de Oliveira *et al.* 1988). Notably, lectins being proteins are heat labile and can be inactivated by precooking of meals (Bender 1983). The role of lectins in soybean meals as an ANF has been implicated, although it is difficult to understand how this is viable given the heat lability of these molecules and the high temperatures involved in oilseed cake drying and cooking extrusion pelleting of feeds (Baeverfjord & Krogdahl 1996; Refstie *et al.* 1998).

Oligosaccharides

Oligosaccharides are carbohydrate molecules of a α-galactosyl homologue of sucrose found in appreciable levels in lupins, rapeseed and soybean meals. Oligosaccharides also contain significant amounts of the raffinose, stachyose, verbascose and sucrose families. Of these, raffinose is a trisaccharide molecule with a single galactose moiety linked to the disaccharide sucrose molecule (formed of glucose and fructose). Stachyose has two galactose moieties linked to the sucrose and verbascose three (Petterson 2000). These are different molecules from mannan oligosaccharides and fructose oligosaccharides, both of which have been used as prebiotics in some animal feeds (Grisdale-Helland *et al.* 2008).

High levels of raffinose oligosaccharides have been reported to present some negative nutritional effects, some of which may be applicable to fish (Glencross *et al.* 2003a). These include; (a) interference with the digestion of other nutrients, (b) osmotic effects of oligosaccharides in the intestine; and (c) anaerobic fermentation of the sugars resulting in increased gas production and other fermentation products. Other studies examining ethanol-soluble carbohydrates (most likely to be oligosaccharides) from soybean meals on Atlantic salmon, have also shown some antagonistic effects (Refstie *et al.* 1998).

Phytate

The molecule inositol-hexaphosphate and salt ions of this molecule are commonly referred to as phytate. The phytate molecule is strongly negatively charged at all pH values usually encountered in feeds. As a consequence of this, phytate has been known to complex with proteins at acidic pH values and also polyvalent ions, such as zinc, at intestinal pH values (Nelson *et al.* 1968).

This complexing of phytate with other nutrients has been attributed to a reduced availability of these nutrients to animals when fed diets with a significant phytate content. It has also been suggested that high dietary calcium levels can exacerbate the complexing of zinc with phytate (Nelson *et al.* 1968; Hardy & Shearer 1985;Richardson *et al.* 1985). Other significant effects that have been attributed to phytate include depressed growth, depressed feed intake, reduced protein utilization and depressed thyroid function (Satoh *et al.* 1989). A dose–response study with channel catfish was undertaken with 0%, 1.1% and 2.2% inclusion of phytate. In this study, the authors observed a reduction in the content of some divalent minerals in the bone of the fish (e.g. zinc) and at higher levels growth was significantly reduced.

The commercial use of exogenous enzyme supplements has made considerable improvements to the utilization of the phosphorus content of phytates by both pigs and poultry (Simons *et al.* 1990). The key to this is the use of the enzyme phytase (EC 3.1.3.8) which cleaves the phosphate units from the inositol base. Studies have indicated that there may be potential for phytase use with fish diets (Teskered *et al.* 1995; Storebakken *et al.* 1998; Mwachireya *et al.* 1999; Sugiura *et al.* 2001; Ai *et al.* 2007; Cao *et al.* 2007).

Protease inhibitors

Protease inhibitors are specific substances that can inhibit the proteolytic activity of certain digestive enzymes.

Protease inhibitors are generally classified according to the type of protease they inhibit, and a range have been identified from a variety of plant meals. Soybeans are well known as a plant meal with a substantial protease inhibitor content and the impact of protease inhibitors from this source have been shown to impact on fish (Dabrowski *et al.* 1989; Krogdahl *et al.* 1994, 2003; Moyano *et al.* 1999). At least five different types of protease inhibitors have been identified in the seeds of soybean, with trypsin inhibitor levels as high as about 34,000 mg/kg DM in unprocessed seed to about 10-fold reduction to 3,400 mg/kg DM in a processed meal (Wilson & Poe 1985). The primary mode of action of protease inhibitors is through either the competitive or allosteric binding of the substance to the digestive enzyme to render it inactive (Krogdahl *et al.* 2010). Like lectins, most protease inhibitors are themselves proteins and are therefore heat labile and can be inactivated by precooking of meals prior to their use.

A dose–response to crude soybean trypsin inhibitor was determined by Olli *et al.* (1994) in Atlantic salmon (*S. salar*). With increasing inclusion of the trypsin inhibitor, the authors reported a decline protein and fat digestibility, a reduction in weight gain and lower trypsin activity in the intestine. The response curve to the varying doses indicated that Atlantic salmon were able to compensate for the presence of small amounts of trypsin inhibitor, but at high levels virtually all endogenous trypsin secretion was depleted. The influence of protease inhibitors from potatoes was examined by Sveier *et al.* (2001) in a dose–response manner. In contrast to the findings with soybean trypsin inhibitor, these authors observed an improvement in growth of Atlantic salmon at low protease inhibitor inclusion levels. However, activities of trypsin, chymotrypsin, carboxypeptidases A and B in different segments of the intestine were all reduced.

Saponins

Saponins are plant glycosides with a steroid or triterpenoid structure as part of the molecule (Francis *et al.* 2002a). Similar to alkaloids, saponins are also a bitter tasting molecule. This means that their primary antinutritional basis is primarily as a feed intake deterrent. An additional effect attributable to saponins is that they increase the permeability of the small intestine mucosal cells (Fenwick *et al.* 1991).

Overall, the effects of saponins have been mixed with some implications of nutritional pathologies in shrimp and salmonids associated with the use of soybean meals in particular (Bureau *et al.* 1998; Francis *et al.* 2001b, 2002b, 2005; Refstie *et al.* 2006; Urán *et al.* 2008, 2009a,b), while in other cases the influence of saponins has been reported to be nominal with European seabass (*Dicentrarchus labrax*; Couto *et al.* 2015). In some studies, saponin inclusion has resulted in improved growth rates (Francis *et al.* 2001b). In this study, a dose–response trial with saponins (0, 150 or 300 mg/kg) found slight growth improvements in a linear response to inclusion when fed to Tilapia (*Oreochromis niloticus*).

Tannins

Tannins are a group of polyphenolic compounds that bind to other proteins to either inhibit their activity in the case of digestive enzymes or to prevent their digestion, in the case of most other proteins (Hagerman *et al.* 1992). Tannins are also bitter tasting compounds known to reduce feed intake by animals (Kumar & Singh 1984). There are two tannin subgroups, those being either the hydrolysable or condensed (non-hydrolysable) forms (Bravo 1998). The condensed tannins have been reported to be able to precipitate proteins, particularly the digestive enzymes. Tannins can form cross-linkages between proteins and other macromolecules and render them unavailable for digestion (Griffiths 1991).

There are few studies on tannins specifically with fish from which to draw dose–response data. One such study was that by Becker and Makkar (1999), with common carp. In this study, the authors examined the dietary inclusion of two different types of tannin (tannic acid and a condensed tannin), each in the diet at 2%. The condensed tannin (included as Quebrancho tannin) had no effect on the fish performance, whereas the tannic acid (a hydrolysable tannin) produced adverse effects within 4 weeks including a reduction in feed intake, poorer growth and a reduction in the metabolic rate (as measured by oxygen consumption).

Other ANF

Another potential limitation, although usually not considered an ANF as such, is the presence in most feed grain products of a certain level of NSP. Effectively, NSP in most grains include the cellulose, hemicellulose and lignin content that forms the structural carbohydrates and it has no nutritional value and acts little more than as filler. However, in some cases, it has been demonstrated that it can interact with other nutrients to diminish their value (Hansen & Storebakken 2006; Kraugerud *et al.* 2007; Sinha *et al.* 2011). The molecule lignin has been identified as playing a particularly negative role in nutrient assimilation by fish (Glencross *et al.* 2008a, 2012; Irvin *et al.* 2016).

To address the issue of NSP levels in feed grains, there are several options. One option is that of physical processing of the grain to remove as much fibre and NSP as possible. This effectively creates protein concentrates with reduced NSP levels. Methods such as air classification and solvent extraction to cost-effectively create such protein concentrates have already been reported and exist as commercially available products (Drew *et al.* 2007a). The review by Drew *et al.* (2007a) comprehensively covers the background information on the nutritional influences of such processing methods. The use of supplementary exogenous enzymes, both as a preliminary processing method of the grain/meal and as a dietary addition has been examined in a few instances. Enzymes that have been examined for use in such scenarios include α-galactosidases and xylanases (Glencross *et al.* 2003a; Stone *et al.* 2003; Ai *et al.* 2007).

Zoonotic threats

Another potential risk associated with feeds and feed raw materials is the potential for the introduction of zoonotic threats – diseases. Indeed, this risk has been implicated as a major 'Achilles heel' in the use of rendered mammalian meals in some parts of the world since the 1990s. However, the type of disease threats, and the risk associated with each of them, varies considerably. In this section of the review, we have broken down the discussion into sections on each of the major agents considered a potential risk; transmissible spongiform encephalopathies (TSEs), microbes (bacteria and viruses) and parasites.

Transmissible spongiform encephalopathies

Transmissible spongiform encephalopathies, also known as prion diseases, are a group of neurodegenerative diseases that affect the nervous system of many animals, including humans (Collinge 2001; Collins *et al.* 2004; Riesner 2004). The disease is characterized by a deterioration in mental and physical abilities of the infected animal, progressing ultimately to death. Following infection, a myriad of tiny vacuoles forms in the brain cortex giving it the appearance like a sponge (hence the term spongiform) (Jeffrey *et al.* 1995). Prion diseases of humans include a spectrum of diseases with overlapping signs and symptoms. The best known of these are Creutzfeldt–Jakob Disease (CJD) and variant Creutzfeldt–Jakob Disease (vCJD) (Will *et al.* 1996; Hill *et al.* 1997; Valleron *et al.* 2001).

In contrast to other kinds of infectious disease, the infectious agent in TSEs is a type of protein called a prion (Collinge 2001). Transmissible spongiform encephalopathies are unique diseases in that their development can originate from genetic, sporadic, or infectious origins (Collinge 2001). For infectious origins of the disease, which is what underpins the primary route of concern in feed risk, it is the ingestion of infected foodstuffs which is the principle path of infection (Collinge 2001). Transmission of TSEs typically occurs when uninfected animals consume contaminated tissue from another animal with the disease. The disease appears to predominate among ruminants, with forms such as scrapie (sheep), chronic wasting disease (CWD; deer) and bovine spongiform encephalopathy (BSE; cattle), each having resulted in a series of epidemics in different countries around the world and caused significant shifts in raw material use in feeds in those regions (Wilesmith *et al.* 1988; Will *et al.* 1996; Valleron *et al.* 2001). It is alleged that these epidemics occurred because the infected cattle were fed the processed products from other ruminants (Colchester & Colchester 2005). However, there is also an inherited form of TSE, which occurs in animals carrying a rare mutant prion allele, which expresses prion proteins that change by themselves into the disease-causing conformation (Richt & Hall 2008).

Transmissibility of TSEs

Transmissible spongiform encephalopathies are a complex group of diseases that are still poorly understood. There are at least six different theories that have been used to explain their cause and transmission (MacKenzie 2007; Manuelidis *et al.* 2007; Schneider *et al.* 2008). Contributing to the difficulty in understanding the disease is the long incubation period (up to 8 years for cattle). Presently, there is no single theory that has been proven to explain the cause of BSE and/or CJD (Foster & Hunter 1998; Colchester & Colchester 2005). Presently, it is clear that prion proteins are involved in the disease (Hill *et al.* 1997). However, their role in the disease, its pathogenicity and infectious nature, are not completely clear. Therefore, it has been difficult to determine whether prion proteins cause the disease or are a symptom of the disease produced by some other unidentified infectious agent or toxin (Doherr 2007).

Evidence to date supports that BSE is not easily passed from animal to animal, and as such it is not considered a contagious disease (Foster & Hunter 1998; Colchester & Colchester 2005). Once an animal is infected it affects specific tissues, predominantly the brain, spinal cord and a few other tissues. Tissues such as muscle and fat do not appear to be affected by the disease. Research has demonstrated that prions cannot be transmitted through aerosol transmission or through skin contact or most other forms of casual contact. However, prions can be transmitted through direct contact with infected tissue, bodily fluids, or contaminated equipment (Foster & Hunter 1998). Standard sterilization procedures such as boiling, autoclaving or irradiating contaminated tissues and/or equipment have been shown to be unsuccessful in rendering prions non-infective. Although heat processing does not destroy the infectious agent, processing at 134°C for 3 minutes did result in a 2.5-fold reduction in infectivity (Schreuder *et al.* 1998). Resistance to heating at up to 600°C has also been reported (Brown *et al.* 2000). Therefore, the risk of spreading BSE by feeding fully processed rendered ruminant meal to ruminants is extremely low, but still possible theoretically (Taylor & Woodgate 2003).

The species of animal used to produce a rendered meal differ in their level of risk. Additional to species is the type of tissue used to produce a rendered animal meal, which also affects the risk from TSE (Foster & Hunter 1998). To date, neither pork nor poultry derived rendered animal meals have been implicated as potential sources of any TSE (Moore *et al*. 2011). Indeed, it has been widely stated that materials derived from non-ruminant animals approved for human consumption constitute little risk for use in animal feeds (Moore *et al*. 2011). Following the peak outbreaks of TSEs in the UK in the early 1990s, the use of all processed animal proteins (PAPs) in animal feeds was banned in the EU (European Commission, 2001). Following a BSE risk assessment by the EFSA Panel on Biological Hazards (BIOHAZ) (2011), the EU recently set out a working plan for the re-authorization of the use PAPs in animal feeds in 2013, initially for aquafeeds (European Commission (EC) 2013).

Of those experiments designed to study the transmission of TSEs among animals, there have been either of two strategies; the transmission from within the same species or from species to species. The method of infection used has also varied, with intracranial, intraperitoneal and oral introduction of raw nervous tissue from infected animals to test animals attempted (Foster & Hunter 1998). Oral transmission of infected material has been assumed to be much less effective than other methods of transmission because of the processes of intestinal absorption followed by transport and requirement for concentration of the infectious agent in the target tissues needing to occur and that this process (digestion) is generally designed to neutralize most zoonotic threats. Therefore, oral exposure is generally considered to be a hundred thousand-fold less effective than direct exposure by the intra-cranial route (Schreuder *et al*. 1998). Given the potential losses that may occur via oral exposure, a large number of infectious units must be consumed in order for the disease to develop. In human cases, it was estimated that the oral infectious dose (ID_{50}) was just over 1000 BSE prion molecules. Notably, this is a relatively large dose compared to that required from known bacterial and viral pathogens (Maignien *et al*. 1999).

The effects of orally or intracranially challenging cattle with rendered proteins and fats from scrapie-infected sheep found no evidence of oral transmission at any time during a long-term study (8 years; Cutlip *et al*. 2001). In a second experiment, cattle orally challenged with rendered scrapie positive brain tissue from sheep were all tested negative for BSE (and scrapie) after 8 years. However, those cattle challenged with intracranially infections did test positive for a scrapie-like infection (Cutlip *et al*. 1994). In another study, cattle were orally challenged with samples of mule deer infected with CWD another type of TSE found in North America. The cattle were inoculated (oral or intracranial) with brain tissue from CWD-infected mule deer and after 2 years animals from each challenge group (oral or intracranial) were tested and found to be negative for the disease (Mathiason *et al*. 2006).

In a study by Moore *et al*. (2011) to determine the susceptibility of chickens to BSE, the birds were challenged with BSE-infected brain tissue by intracranial, intraperitoneal and oral routes. Interestingly, no infection was observed in any of the chicken tissues assayed at the end of the study, regardless of the route used to introduce infective material. It was judged that these results supported the notion that BSE transmission to avian hosts is not viable.

There have been few studies on transmissibility of TSEs to fish (Dalla-Valle *et al*. 2008; Ingrosso *et al*. 2006; Salta *et al*. 2009). A study using rainbow trout (*Oncorhynchus mykiss*) and turbot (*Scopthalamus maximus)* examined the potential transmissibility of TSEs to fish (Ingrosso *et al*. 2006). In that study, the fish were fed a mouse adapted strain of scrapie (139A), and both fish species showed an ability to clear the majority of the infectious load. None of the tissues sampled following feeding were able to induce scrapie disease in mice via the most reliable route of intracerebral inoculation. These authors also assessed whether the prion protein could cross the intestinal epithelium using an *in vitro* assay and found that the prion proteins bound to the mucosal side of the intestine and that there was effectively no active uptake of the prion protein across the intestinal wall. A similar study by Dalla-Valle *et al*. (2008) examined whether a specific type of TSE, an abnormal prion protein (PrP^{sc}) could cross the intestinal barrier of rainbow trout. Their observations noted that the PrP^{sc} were absorbed by the intestinal mucosa within 3 days and persisted in the pyloric caeca up to 7 days, but by 15 days no detectable levels remained. Notably, none crossed the intestinal barrier. In a third study where Gilthead seabream (*Sparus aurata*) were fed neural tissue from BSE-infected cattle or scrapie-infected sheep, none of the fish developed any clinical signs of a prion disease (Salta *et al*. 2009). However, the authors did conclude that 2 years after feeding the TSE-infected tissue to the fish that the brain tissue of those fish did show signs of neurodegeneration and accumulation of deposits that cross-reacted with antibodies raised against endogenous seabream prion proteins. Importantly, substantial differences were observed between fish fed either BSE- or scrapie-infected tissues, with the BSE-fed fish showing greater signs of neurodegeneration than the scrapie fed fish. Between these two studies, the contrasting results raise some important questions about methodology and/or species implications.

Control of TSEs

To manage the spread and minimize the impact of TSEs, various government organizations around the world have implemented a range of biosecurity measures. In the United States, they have developed what is being referred to as the 'triple firewall strategy' which was implemented to prevent BSE from occurring within the country. The United States programme is one designed to proactively prevent the introduction of BSE (using defined import restrictions), limit the amplification of any outbreaks should the disease occur and implement a comprehensive surveillance system. In 2004, the United States Department of Agriculture (USDA) initiated a testing program to determine the incidence of BSE in the United States. Over a 2-year period, the USDA tested 787,711 cattle and found just two BSE positive cases. An analysis of 7 years of surveillance data identified that the estimated prevalence of BSE in the United States to be less than one infected animal per one million cattle (BSEinfo, 2014).

One element of risk management for TSEs is the effective identification of raw materials. Presently, several research groups around the world are working to develop testing methodologies to assist in the identification of the type of material from which animal proteins are derived. It has been demonstrated that it is possible to identify species-specific DNA using polymerase chain reaction (PCR) even if the DNA from the sample is partially degraded (Dalmasso *et al.* 2004; Lahiff *et al.* 2001). Using an enzyme-linked immunosorbent assay (ELISA), it is also possible to differentiate skeletal muscle in protein meals, from other tissues (Schmidt *et al.* 1999). A test for normal brain prion protein (which is the progenitor for the disease related form of prion) has claimed the ability to detect these molecules at one part in a hundred billion using a method called Surround Optical Fibre ImmunoAssay (SOFIA) and specific monoclonal antibodies raised against the brain prion protein from hamsters, sheep and deer (Chang *et al.* 2009).

It has been recognized that there are also regional differences in the risk associated with TSEs (EC, 2013; FSANZ, 2014). It is argued that animal protein sources from countries where BSE has never been reported represent a much lower level of risk than countries where the disease has previously been reported. Accordingly, different countries are given one of three distinct 'categories', ranking from category 1 (negligible BSE risk) and category 2 (controlled BSE risk) and category 3 (undetermined BSE risk) which is maintained for those countries with a history of BSE and/or in ability to demonstrate requirements for categories 1 or 2. As such, BSE largely remains a regional disease and is presently confined to the EU (including United Kingdom) and North America (Chesboro 2004; Hill *et al.* 1997; Richt & Hall 2008). In the case of Japan, the only country outside the EU or United States to report a non-imported outbreak of BSE, the cattle that tested positive were assumed to have contracted the disease through eating infected meat and bone meal that originated from the EU (Kamisato 2005). Australia and New Zealand are both considered free of these diseases. To maintain this status, as well as their ability to confidently use ruminant protein meals in some monogastric feeds, both countries rely on controlling import of animal meals, strict feeding regulations and proactive surveillance measures (FSANZ, 2014).

Microbiological agents (Bacteria and Viruses)

Transfer of microorganisms, such as bacteria and viruses from feed/food to host has been long recognized as a major mechanism of disease transmission (Crump *et al.* 2002; Dalsgard 1998; Dee *et al.* 2014). Conversely, in some instances, the intended contamination of feed with a probiotic live culture of bacteria has shown some beneficial effects in some species as well (Gatesoupe 1999; Wang *et al.* 2008). So not all microbiological contamination can be considered necessarily a negative aspect of feeds. Of those bacteria linked to negative aspects of feed sanitation, there are a range of bacterial species that have been associated with disease transmission to either the animals to which they are fed, or through subsequent promulgation through the food chain to the human consumers (Cabello 2006; Dalsgard 1998; Huss 1997; Metcalf *et al.* 2011). Key bacterial species in this group include the *Clostridia, Listeria, Campylobacter* and *Salmonella* species.

While transmission of viruses via the diet have been recognized as important causes of outbreaks of some food-borne diseases (e.g. noroviruses and hepatitis A), those foods that have been implicated tend to be those that are eaten raw or minimally processed such as bivalve molluscs, rather than processed foods (Potasman *et al.* 2002; Tei *et al.* 2003; Meng 2011; Kamar *et al.* 2014). Complicating the assessment of food borne virus risk is the fact that they tend to be underdiagnosed and underreported in the regions where they are known to be prevalent as the analytical and diagnostic tools for such viruses are not widely available. Much progress has however been made in recent years in developing the methodology available for detection and identification of viruses in both food and clinical samples (Nainan *et al.* 2006).

Management of the microbiological risk is considered a dynamic process. A range of data sources and decision-making frameworks have been used. Notably, the parameters of importance have in some cases changed over time (Dennis *et al.* 2002). In 2000, a joint FAO/WHO expert panel on Microbiological Risk Assessment (JEMRA) was initiated in response to a request from the Codex Alimentarius Commission and various FAO and WHO Member countries. At this time, it is was considered that there was an increasing need for scientific advice on the risk derived from microbiological food safety issues (World Health Organization, 2003).

The aim of JEMRA was to devise and optimize a framework on the utility of Microbiological Risk Assessment (MRA) as a potential tool to inform decisions with the objective of improving food safety in both developing and developed countries. However, several limitations to the use of such an MRA have been identified. First, there needs to be an initial process that brings key issues into focus and subsequently guides further action in a systematic manner that allows risk managers to do so on a consistent basis (Buchanan 2004). It has been acknowledged that using such an MRA in feed/food safety risk management is an area that is still developing (Karunasagar 2016).

Contamination via raw materials

The principle route of microbial contamination of animal feeds is via the use of certain types of raw materials (Isa *et al.* 1963; Sapkota *et al.* 2008). Although all types of feed materials have been assessed and various levels of microbial contamination encountered, it has been the rendered animal (marine and terrestrial) meals that have generally been considered the highest risk (Lunestad *et al.* 2007; Sapkota *et al.* 2008).

Salmonella has been considered one of the more important pathogenic bacterial species to manage in feed raw materials (Burr & Helmboldt 1962; Veldman *et al.* 1995; Lunestad *et al.* 2007). In a published international survey of various types of protein resources, mixed results were obtained as to the level of contamination across animal, plant, grain and fish protein resources (Table 4) (Isa *et al.* 1963; Brooks 1989; Sreenivas 1998; Beumer & Van der Poel 1997). Based on this assessment, it can be noted that there are clearly wide levels of variation across geographical regions and raw materials and that there was a lack of consistency in which materials had high or low levels of contamination.

A survey of Norwegian fish feed production facilities found that at that time less than 4% of the examined environmental samples contained *Salmonella* and the prevalence in the fish feeds themselves was reported at only 0.3% of samples (Lunestad *et al.* 2007). These authors suggested that the risk of transmission to humans from fish products was minimal. Additionally, there was no evidence to suggest any transmission from the feed to humans.

Table 4 Incidence (% of samples contaminated) of Salmonella in feed raw materials

Raw material	Netherlands a	Germany b	UK c	USA d	Canada e
Animal proteins	6	6	3	33	20
Plant proteins	3	26	7	10	18
Grain	–	3	1	0	5
Fishmeal	–	–	22	10	22

Data derived from aBeumer and Van der Poel (1997); bSreenivas (1998); cBrooks (1989); dIsa *et al.* (1963); [e]Canadian Food Inspection Agency (2005).

Extending from this assessment an MRA risk factor approach to using various raw materials was proposed by Brooks (1989). This entailed multiplying the inclusion level of a specific raw material by the intrinsic MRA of that raw material (e.g. Table 5). Using this approach, over time the MRA risk of feeds for microbiological factors such as *Salmonella* can be considered to have reduced by about half. This has occurred principally through a reduction in the use of higher risk raw materials such as fishmeal and an increase in the use of lower risk raw materials, such as plant proteins.

Contamination during processing and storage

The processing of raw materials and compound feeds provides a clear opportunity for management of the MRA for a range of potential microbiological contaminants. Many of the processes used in processing raw materials involve various uses of solvents and/or heat and in many cases such processing can reduce the level of microbiological contaminants (Drew *et al.* 2007a).

Processing of animal (both terrestrial and marine) proteins (rendering) has been one raw material sector that has spent considerable effort to review the potential for microbiological risk transfer (Malicki *et al.* 2005; Sapkota *et al.* 2008). In a study examining the contamination levels of four different classes of microbiological contaminants (*Clostridium, Listeria, Campylobacter* and *Salmonella*) in raw tissue and post-processing, the authors found that none of the materials retained any contamination after the rendering process. In some cases, more than 80% of raw tissue material samples were found to be contaminated, but irrespective of this none of the post-processed materials retained any contamination.

After processing of either the material or feed product, there remains the potential for microbiological contamination under certain environmental conditions (Duncan & Adams 1972; Carrique-Mas *et al.* 2007). To mitigate this, organic acids and other preservatives are often applied to

Table 5 Relative risk of Salmonella contamination in complete feeds based on the method proposed by Brooks (1989) when applied to data from Ytrestoyl *et al.* (2015) of changing formulation practices in the Norwegian salmon feed industry from 1990 to 2010

Atlantic salmon feed – 1990	Amount in formula (%)	Salmonella Incidence (%)	Risk factor
Grain	9.6	0.9	0.086
Plant protein	0.0	2.7	0.000
Fishmeal	65.4	13.2	8.633
Oils	24.0	0	0.000
Micro-ingredients	1.0	0	0.000
		Total	8.719
Atlantic salmon feed – 2000	**Amount in formula (%)**	**Salmonella Incidence (%)**	**Risk factor**
Grain	11.2	0.9	0.101
Plant protein	22.2	2.7	0.599
Fishmeal	33.5	13.2	4.422
Oils	31.1	0	0.000
Micro-ingredients	2.0	0	0.000
		Total	5.122
Atlantic salmon feed –2010	**Amount in formula (%)**	**Salmonella Incidence (%)**	**Risk Factor**
Grain	8.4	0.9	0.076
Plant protein	35.5	2.7	0.959
Fishmeal	24.8	13.2	3.274
Oils	29.1	0	0.000
Micro-ingredients	2.2	0	0.000
		Total	4.308

reduce the water activity level and thereby reduce the potential for establishment of microbiological and/or fungal contamination.

Parasites

Parasitic organisms are defined as those organisms that live in or on another organism without benefiting the host organism. Typically, these organisms are considered pathogenic to their host. The most common parasites encountered via dietary vectors are usually protozoans and helminths (Seng *et al.* 2006; Rückert *et al.* 2009). However, for such parasitic infections to be transferred from feed to host, there needs to be little to no processing of the feed. The use of natural feeds, like bait-fish (trash-fish), as still occurs as a key feed resource in many developing regions of the world, provides a likely vector for parasitic infection of the fish to which they are being fed (Rückert *et al.* 2009; Kim *et al.* 2013). By contrast, the routine drying, milling and feed processing used to make manufactured feeds have been shown to kill most if not all potential parasitic agents (McDonnell 2007).

Impact of feed processing on sterilization

Most microbiological and parasitic vectors, being living organisms, are sensitive to various processing techniques that have the impact of sterilizing the material in which they are contained (Okelo *et al.* 2008). Feed extrusion, the main method by which most modern aquaculture feeds are now made, has been shown to be highly effective in reducing microbial contamination (Kelley & Walker 1999; Okelo *et al.* 2008; Bianchini *et al.* 2012). During the extrusion process, it is quite typical for the feed extrudate temperature to exceed 100°C concomitant with high levels of pressure and shear stress. A similar, but less extreme effect in reducing bacterial contamination of feeds has also been noted on steam-pelleted feeds fed to poultry (Furuta *et al.* 1980). However, feeds can be contaminated after feed processing and thermal sterilization while being conveyed in feed plants, shipped or stored. It is important to note that feeds are not sterile by the time they are fed to fish but rather contain robust and diverse microbial communities.

Genetically modified organisms

A genetically modified organism (GMO) or transgenic organism is one whose genome has been altered using genetic engineering techniques in contrast to other genetic approaches such as more traditional selective breeding programmes. The Cartagena Protocol on Biosafety to the Convention on Biological Diversity, an international agreement to protect biological diversity from the potential risks posed by organisms resulting from modern biotechnology uses the term ‘Living Modified Organisms’ (LMO), although this is regarded as equivalent to GMO (Secretariat of the Convention on Biological Diversity, 2000). As well as being a research tool, GMOs have practical and commercial applications in the production of pharmaceutical drugs, experimental medicine (e.g. gene therapy) and agriculture. Examples in agriculture include increasing production yields through, for instance resistance to herbicides, and also improving nutrient levels such as the vitamin A precursor β-carotene in golden rice (Ye *et al.* 2000). The global area of genetically modified (GM) crops has increased 100-fold in the past 19 years and in 2014 around 12% of the world’s crops were GM varieties with more than 18 million farmers in 28 countries worldwide growing GM crops (ISAAA, 2014). The USA produces around 40% of the global production of GM crops, with 95% sugarbeet, 93% of the cotton, 93% of the soybeans and 86% of the corn grown in that country being GM varieties, and 95% of the canola/rapeseed crops grown in Canada are now GM

(International Service for the Acquisition of Agri-Biotech applications (ISAAA) 2014). Despite the perception that Europe is a GM-free zone, five countries in the EU grow GM maize (International Service for the Acquisition of Agri-Biotech applications (ISAAA) 2014).

The increasing use of plant ingredients derived from terrestrial agriculture in fish feeds means that that GM materials are also being increasingly used. This is particularly the case for soybean (meals and oils) and maize (meal) products but also, to a lesser extent, cotton (meal) and canola/rapeseed (oil) products. While the use of ingredients containing GM materials in fish feeds is accepted practice in North and South America and Asia, they are currently not used in aquaculture feeds in Europe. This is not driven by legislation as GM-crops can be used for human food consumption and in animal feeds if approved by the EU and labelled per EU legislative requirements, thus around 80% of feeds for livestock in the EU contain GM crops (SARF, 2015). The situation with fish feeds is market driven based on perceived consumer resistance and consequent retailer demands and, as a result, fish feeds produced in Europe are non-GM according to relevant EU labelling regulations (Scottish Aquaculture Research Forum (SARF) 2015). Currently, to satisfy EU legislations (1829/2003 and 1830/2003), a threshold labelling at 0.9% for the adventitious presence of approved GM material is required (European Commission, 2003a,b). However, due to the widespread production of GM crops, it is becoming increasingly difficult for fish feed manufacturers in Europe to source non-GM raw materials for fish feed and so non-GM feed may become impossible to guarantee, which would prompt a necessary change in policy in the European aquaculture, feed and food sectors (Shepherd *et al*., 2017).

Although there have been countless studies investigating the replacement of marine ingredients with plant products relatively few have focused on the possible GM origin of the ingredients or on determining the specific effects of GM versus the equivalent non-GM ingredients (Table 6; Padgette *et al*. 1995; Hammond *et al*. 1996; Brown *et al*. 2003; Glencross *et al*. 2003b; Sanden *et al*. 2004, 2006; Hemre *et al*. 2005, 2007; Chainark *et al*. 2006; Sagstad *et al*. 2007, 2008; Sissener *et al*. 2009). In assessing GM products in fish feeds there are two main areas of possible interest and/or concern including (i) production – does the GM product alter the growth performance (growth rate and feed efficiency) of the fish, and (ii) safety – does the GM product affect fish health or welfare and/or the safety of the farmed product. Early studies investigated the effect of glyphosate-tolerant GM soybean in feed for catfish on growth performance in terms of final fish weight and concluded that the feeding values of the GM soybeans were not different to non-GM soybean (Padgette *et al*. 1995; Hammond *et al*. 1996). Subsequently, various GM plant products in several fish species have been evaluated including methionine-enhanced GM lupin in red seabream (*Pagrus auratus*; Glencross *et al*. 2003b), glyphosate-tolerant canola in rainbow trout (*Oncorhynchus mykiss*; Brown *et al*. 2003) and Bt maize in Atlantic salmon (Sanden *et al*. 2006; Hemre *et al*. 2007). Other than reduced growth related to a mild stress response in one trial (Hemre *et al*. 2007; Sagstad *et al*. 2007), generally no negative effects on growth were reported. In a long-term trial, Atlantic salmon were fed genetically modified soy and growth performance and general health monitored, with the authors concluding that the effects of feeding GM soy were minor, and lack likely caused by variations in the soy strains rather than the genetic modification per se (Sissener *et al*. 2009). Regarding food safety, one possible issue could be whether transgenic sequences can be transferred to the fish and found in tissues including muscle and thereby possibly further transferred to human consumers. One study investigating GM soy in feeds for Atlantic salmon demonstrated that transgene sequences may survive passage through gastrointestinal tract but were not found in salmon tissues (Sanden *et al*. 2004).

So far, the GM materials tested in fish have been developed for agricultural/agronomic purposes, but GM technology can be applied to specifically tailor crops for aquaculture through reduction in antinutritionals and/or modification of the levels of nutrients such as essential amino and fatty acids (Glencross *et al*. 2003b; Gatlin *et al*. 2007). Improving nutrient levels in crops is currently an area of great interest to aquaculture and fish feeds specifically in relation to the provision of the n-3 LC-PUFA, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The n-3 LC-PUFA are important nutrients with key metabolic and functional roles in fish and humans but they are only present in fish oil and fishmeal. Therefore, the major problem in replacing fish oil in feeds is maintaining n-3 LC-PUFA in farmed fish at the high levels required for the farmed products to retain their role as beneficial and healthy components of the human diet. As fish oil and meal are finite and limited resources, this implies that the current global supply of n-3 LC-PUFA is similarly limited and there is a significant gap between supply and demand indicating a fundamental, global lack of n-3 LC-PUFA to supply all human needs, whether by direct consumption or via aquaculture (Naylor *et al*. 2009; Scottish Aquaculture Research Forum (SARF) 2015; Tocher 2015). It is unlikely that microalgal biomass can be produced on the scale necessary and at an economic cost to satisfy the demands of aquaculture for n-3 LC-PUFA, at least in the short- to medium-term (Miller *et al*. 2011; Chauton *et al*. 2015). However, as the primary producers, microalgae represent a highly valuable source of genes encoding for the biosynthetic enzymes required for n-3

Table 6 A summary of studies undertaken on the use of genetically modified raw materials in aquaculture species

Raw material	GM purpose	Test species*	Nutritional impacts on fish	Health impacts on fish	Authors
Corn (Maize)	Insect resistance (Bt gene)	Atlantic salmon	No major impacts	No nutritional pathologies	Sanden *et al*. (2006)
	Insect resistance (Bt gene)	Atlantic salmon	No major impacts	Minor effects on glucose transport and intestinal maltase activity	Hemre *et al*. (2007)
Rapeseed (Canola)	Glyphosate resistance	Rainbow trout	No difference to parental Canola line	No apparent health problems	Brown *et al*. (2003)
Soybean	Glyphosate resistance	Channel catfish	No nutritional impacts	No GM-related effects on health reported	Hammond *et al*. (1996)
	Glyphosate resistance	Atlantic salmon	No major impacts	No nutritional pathologies	Sanden *et al*. (2006)
	Glyphosate resistance	Atlantic salmon	No nutritional impacts	Lowered plasma TAG and slightly enlarged spleen but no major impacts on health	Sagstad *et al*. (2007)
	Glyphosate resistance	Atlantic salmon	No nutritional differences between fish fed GM or non-GM	No differences in health status	Sissener *et al*. (2009)
	Production of 18:4n-3 (SDA)	Rainbow trout	Increased SDA and 20:4n-3 in fillet	No health effects reported	Nanton *et al*. (2012)
	Production of 18:4n-3 (SDA)	Rainbow trout	Increased SDA in flesh	No health effects reported	Park *et al*. (2017)
	Production of 18:4n-3 (SDA)	*Seriola rivoliana*	Increased SDA in flesh	No health effects reported	Park *et al*. (2017)
Lupin	Insertion of gene to upregulate methionine content of seed	Red seabream	Increased level of available methionine	None reported	Glencross *et al*. (2003b)
Camelina	Production of n-3 LC-PUFA in seed oil (EPA only)	Atlantic salmon	Deposition of EPA in tissues	No negative effects reported	Betancor *et al*. (2015a,b)
	Production of n-3 LC-PUFA in seed oil (EPA + DHA)	Atlantic salmon	Deposition of EPA and DHA in tissues	No negative effects reported	Betancor *et al*. (2016a)
	Production of n-3 LC-PUFA in seed oil (EPA + DHA)	Atlantic salmon	Deposition of EPA and DHA in tissues	No negative effects reported	Betancor *et al*. (2017)
	Production of n-3 LC-PUFA in seed oil (EPA only and EPA + DHA)	Gilthead seabream	Deposition of EPA and EPA + DHA in tissues	Reduced growth and increased lipid vacuoles in liver cytoplasm in bream fed EPA-only oil	Betancor *et al*. (2016b)
Yeast (*Yarrowia lipolytica*)	Production of n-3 LC-PUFA in yeast cells (EPA only)	Atlantic salmon	Deposition of EPA in tissues	No negative effects reported	Hatlen *et al*. (2012)
	Production of n-3 LC-PUFA in yeast cells (EPA only)	Atlantic salmon	Deposition of EPA in tissues	No negative effects reported	Berge *et al*. (2013)

*Atlantic salmon (*Salmo salar*), Kampachi (*Seriola rivoliana),* red seabream (*Pagrus auratus*), gilthead seabream (*Sparus aurata*), rainbow trout (*Oncorhynchus mykiss*), channel catfish (*Ictalurus punctatus*).

LC-PUFA production (Venegas-Caleron *et al*. 2010). The overall strategy is to genetically modify existing organisms that have oil deposition as a major trait and thus combine this with the n-3 LC-PUFA biosynthesis trait. Potential candidates include other oleaginous microorganisms or conventional oilseed crops to produce entirely novel sources of *de novo* n-3 LC-PUFA (Zhu *et al*. 2010; Sayanova & Napier 2011).

Progress into the metabolic engineering of oleaginous microorganisms to produce n-3 LC-PUFA has been reviewed recently (Gong *et al*. 2014). The most successful to date has been the metabolic engineering of the yeast

(*Yarrowia lipolytica*), which resulted in a strain that produced EPA at 15% of dry weight (Xue *et al.* 2013). It was shown that the EPA-*Yarrowia* cell mass was suitable as a feed ingredient for Atlantic salmon (Hatlen *et al.* 2012) although disruption of the yeast cell walls was required to increase the bioavailability of lipid and EPA (Berge *et al.* 2013). To the best of the authors' knowledge, the transgenic yeast oil or cell biomass are not yet fully commercially available although they appear to be used by a DuPont/AquaChile venture (Verlasso®) to produce a niche salmon product (http://futurefood2050.com/turning-yeast-into-sustainable-fish-food/). However, as the production of the transgenic yeast uses biofermentor technology, it appears unlikely that it could be produced in volumes and at a cost that would make it viable as a large-scale alternative to fish oil in aquaculture, at least in the short- to medium-term.

Oilseed crops dominate world oil production and there is a highly organized and well-established infrastructure for the cultivation, harvest, processing, distribution, marketing and utilization of vegetable oils (Salunkhe *et al.* 1992). Therefore, oilseed crops are highly practical platforms from which to develop a novel, renewable supply of n-3 LC-PUFA. However, conventional plant breeding strategies cannot be used as the genes required for LC-PUFA synthesis are simply not present in higher plants, leaving transgenesis as the only option for modification of oilseeds to contain LC-PUFA. Therefore, arguably the only currently viable approach to developing a novel, renewable supply of EPA and DHA is the metabolic engineering of oilseed crops with the capacity to synthesize n-3 LC-PUFA in seeds (Haslam *et al.* 2013). The production of n-3 LC-PUFA in terrestrial plant seeds was demonstrated in the model plant Arabidopsis (Petrie *et al.* 2012; Ruiz-Lopez *et al.* 2013), and recently reported in an oilseed crop, *Camelina sativa* (Petrie *et al.* 2014; Ruiz-Lopez *et al.* 2014, 2015). *Camelina sativa* or false flax, is a member of the Brassicaceae family and an ancient crop that, in the wild-type, produces an oil with α-linolenic acid (18:3n-3) at up to 45% of total fatty acids (Gunstone & Harwood 2007). Transgenic *C. sativa* lines have now been developed by transformation with algal genes encoding the n-3 LC-PUFA biosynthetic pathway and expression restricted to the seeds via seed-specific promoters to produce oils with up to 20% of total fatty acids as n-3 LC-PUFA, either as EPA alone or as EPA + DHA (Ruiz-Lopez *et al.* 2014). The transgenic *Camelina* producing the EPA-only oil successfully passed initial field trial evaluation (Usher *et al.* 2015) and the oil has been used to replace fish oil in feeds for Atlantic salmon and was shown to successfully maintain both growth performance and tissue n-3 LC-PUFA levels without any apparent effects on fish health or welfare (Betancor *et al.* 2015a,b). Other GM oilseeds producing n-3 LC-PUFA are being developed including transgenic rapeseed (canola) producing mainly DHA in Australia (Kitessa *et al.* 2014; CSIRO, 2015).

As well as being easily transformable by Agrobacterium floral infiltration, *Camelina* has additional desirable traits including modest input requirements (water and pesticides) and ability to thrive in semi-arid conditions (Tocher *et al.* 2011). In the US, several states are actively growing *Camelina* as a biofuels crop, indicating the wide acceptance of this crop platform. Furthermore, wild-type *Camelina* oil has already been shown to be suitable for inclusion in fish feeds and contains no ANF detrimental to fish growth (Petropoulos *et al.* 2009; Morais *et al.* 2012; Hixson *et al.* 2014). Ultimately, all animal production will depend on terrestrial plants/agriculture and this requires land. However, it is pertinent to emphasize that the production of n-3 LC-PUFA in terrestrial oilseed crops should not require additional arable land as the ideal solution would be to switch some vegetable oil production from n-6 PUFA-rich crops to the new n-3 LC-PUFA crops. The GM oilseed crops represent a novel use of GMO in aquaculture and, upon national and international approvals as new feed ingredients, they are likely to be used in fish feeds in many parts of the world further challenging current attitudes and practice in Europe (Scottish Aquaculture Research Forum (SARF) 2015).

Changes to product qualities

A persistent concern associated with the use of alternative ingredients in feeds for farmed fish is their potential effects on the safety and quality of farmed fish products to the consumer. In terms of the use of alternative ingredients in fish feeds, product safety issues are limited to potential contamination of ingredients with heavy metals or organic pollutants. To the general public, quality of fish products refers to freshness, but in the context of the topic of this review, quality refers to sensory or organoleptic properties, and nutritional properties.

Sensory characteristics

Sensory attributes of fish are those detected using the senses, namely sight, taste, smell and touch. Sensory evaluation of fish products therefore focuses on colour, taste, odour and texture. Each of these attributes can be affected by diet fed to fish. Sensory characteristics of various foods, including fish products, are typically assessed by taste panels, but instruments can also be used to assess colour, odour and texture. Data from Instrumental analysis are considered to be quantitative rather than qualitative (or semi-quantitative) as is the case for data obtained by taste panels. Instrumental analyses are also more sensitive than human senses and therefore can distinguish differences between fish samples that are not detected by human senses. While this is useful to researchers, the importance of such differences at the consumer level is less certain.

Sensory evaluation of fish products using taste panels is generally conducted by triangle testing using untrained evaluators or by organoleptic evaluation by trained panel members who rank samples using specific descriptors. Triangle testing involves presenting three samples to panel members, two being from the same treatment group and one being from a different treatment group. Panellists are asked to choose which sample is different from the other two samples. A variation on triangle testing involves a similar setup but panellists are asked which sample they prefer. Control and treatment samples are mixed and presented to panellists in various combinations, such as two control samples and one treatment sample, or two treatment samples and one control sample. If they consistently select the odd sample as 'preferred' the results are considered robust. Efforts are made to prevent panellists from identifying samples based on appearance unless colour is an attribute being evaluated.

Testing samples using organoleptic evaluation involves more elaborate protocols. Panellists are trained in advance of testing by being presented with samples and specific descriptors, and asked to rank descriptors on a scale, usually 1-9. Descriptors often have colourful names, such as bilgy, briny, fishy, mealy, metallic, mouldy, mushy, musty, putrid, rancid, rubbery and slimy. Of course, there are positive descriptors as well. Each descriptor has a specific definition, and during training panellists are presented with samples that illustrate each descriptor. By this approach, panellists are taught to distinguish samples using descriptors and arrive at a consensus as to what each descriptor represents in terms of sensory attributes. They are also taught to rank the intensity of each descriptor, with a score of one meaning the absence of the descriptor in a sample and nine being highly intense. There are many other elements involved with protocols to conduct organoleptic evaluation and readers are referred to the FAO document repository, specifically to 'Appendix II: Draft guidelines for the sensory evaluation of fish and shellfish in laboratories' for a complete description of protocols. Evaluations are usually made on cooked products, often after refrigerated or frozen storage. The point of the foregoing discussion is to inform readers that sensory evaluation of fish products is complex. Readers must also appreciate the fact that sensory evaluation of fisheries products is not an exact science and that comparing sensory characteristics of fisheries products from different studies can be confounded by the use of different protocols, scoring systems and other factors. Despite this limitation, trends associated with alternate ingredient use in feeds on sensory characteristics of farmed fish are clear.

Although researchers have documented definite effects of alternative protein and oil sources on sensory characteristics of farmed fish products, for the most part the effects are relatively minor and primarily confined to effects of replacing fish oil with alternative oils (Rasmussen 2001). The first report on this topic was by Dupree *et al.* (1979) and involved assessment of flavour using specific descriptors by a trained taste panellists. Significant differences flavour of channel catfish were associated with dietary oil source and level. Catfish fed diets containing increasing levels of menhaden oil or corn oil could be easily distinguished once added oil levels exceeded 5%, with panellists preferring fish fed corn oil diets. Subsequent studies generally support this finding that replacing part or all fish oil in fish feeds with alternative plant oils (soy, canola, rapeseed, sunflower) or animal fat (poultry, swine) alters fillet odour/flavour, and is associated with an increase in consumer/panellist preference in trout (Boggio *et al.* 1985; Smith *et al.* 1988; Guillou *et al.* 1995; Skonberg *et al.* 1998;Turchini *et al.* 2003), salmon (Hardy *et al.* 1987; Thomassen & Rosjo 1989; Bjerkeng *et al.* 1997;Koshio *et al.* 1994; Torstensen *et al.* 2005), gilthead seabream (Izquierdo *et al.* 2005) red seabream (Glencross *et al.* 2003c), sea bass (Montero *et al.* 2005) and turbot (Regost *et al.* 2001).

Fillet colour is an important attribute for salmon and trout, and external colour is also an important attribute in some markets for fish, such as the red sea bream, *Pagrus major/P. auratus* (Booth *et al.* 2004; Choubert & Storebakken 1989). For farmed shrimp, colour after cooking is also an important market factor. Colour is measured by making a visual comparison to standard colour hues and intensities using the Roche *Salmo*fan™, or by using an instrument that measures brightness, hue and colour, such as the Minolta ChromaMeter™ (Skrede & Storebakken 1986). Skin, muscle and egg colour results from deposition of carotenoid pigments supplied in the diets of wild or farmed fish. Alternative ingredients containing the carotenoid pigment astaxanthin, such as krill meal, shrimp meals or their oils, enhance the colour of fillets and skin. Alternative ingredients can also negatively affect colour, but mainly in white-fleshed trout. Corn gluten meal contains xanthophyll, a yellow carotenoid pigment. Feeding corn gluten meal imparts a yellow hue to white-fleshed trout (Skonberg *et al.* 1993). High levels of poultry fat can have the same effect if poultry have been fed feeds contain corn as a major ingredient. Species of fish that do not deposit carotenoid pigments in their flesh are not affected by the presence of xanthophyll or other carotenoid pigments in their diet.

Texture is an important sensory characteristic that is evaluated by trained taste panels, with descriptors such as greasy, soft, chewy, grainy and firm being typical descriptors. Texture is also measured using instruments, such as an Instron probe, that measure the force required to compress fillets a given distance. Although texture is regularly assessed by sensory evaluation of fish products, almost all studies in which fish fed diets with alternative ingredients report no significant differences associated with diet. Texture, defined as firmness, is negatively correlated with muscle fibre diameter (Hatae *et al.* 1990) and by muscle fibre density, meaning number of muscle fibres in a given cross-sectional area (Johnson 1999; Johnson *et al.* 2000). Muscle fibre numbers in fish can be affected by feeding level at specific life history stages in fish (Kiessling *et al.* 1991; Johansen & Overturf 2006). However, there are no reports documenting changes in muscle fibre associated with the use of alternative ingredient. In fact, a number of studies in which fish, mainly rainbow trout, have been fed diets in which fishmeal has been replaced with plant proteins report no differences in a range of sensory attributes, including texture.

Nutritional qualities

The nutritional qualities of fisheries products are associated with their nutritional profiles, namely the contents of protein, fat, vitamins and minerals. Protein and amino acid contents of fish muscle are essentially the same in wild and farmed fish and not affected by feed composition. Muscle amino acid content is associated with the major proteins in muscles, for example actin and myosin, and the amino acid contents of these proteins are conserved in vertebrates, including fish species. Thus, feed ingredient composition has essentially no effect on protein or amino acid profiles of fisheries products (Kaushik *et al.* 1995; Rasmussen 2001) even though the amino acid profiles of plant proteins and some animal protein ingredients, for example blood meal or feather meal, differ greatly from that of fishmeal. While the per cent protein in fillets changes with season, fish size and life stage, alternate feed ingredients are not a factor in these changes.

The situation is very different for fillet lipid content and fatty acid composition, especially in fish that store lipid in muscle tissue, such as salmon and trout. Fillet lipid content increases gradually in fish as they grow and can be altered by feed intake, dietary lipid content and protein:lipid or protein:energy ratio. However, there is very little difference in digestibility of various lipid sources to fish and therefore little effect of dietary lipid source on fillet lipid level. As a result, alternative lipid sources have little effect on nutritional quality as far as fillet lipid content is concerned, even though fillet lipid content is an important factor in sensory quality assessment.

Fatty acid content is another matter. For over four decades, it has been well known that the fatty acid profile of fish reflects that of their diet, both in wild and farmed fish (Turchini *et al.* 2009). Farmed fish consuming a feed containing fish oil have fatty acid compositions similar to that of fish oil and therefore similar to wild fish. Fish oils used in feeds are produced from several fish species, such as menhaden, anchovy oil, capelin, herring and tuna, and differ somewhat in fatty acid profile. Of importance to the nutritional quality of fish products is the content of long-chain, polyunsaturated omega-3 fatty acids, specifically EPA and DHA, as well as the content of omega-6 fatty acids, notably linoleic acid (C18:2). Differences in EPA and DHA content result from using different fish oils in feeds (Stone *et al.* 2010). However, these differences are minor compared to the effects of replacing a portion or all of the fish oil in fish feeds with plant oils or animal fats (Sales & Glencross 2011). Doing so lowers the content of EPA and DHA in the diet leading to a reduction in levels of these fatty acids and an increase in other fatty acids in fillets (Nichols *et al.* 2014; Sprague *et al.* 2016). Although fatty acid profiles of fish fillets reflect that of their diet, the relationship between dietary fatty acids and categories of fatty acids is not exact because fish possess the ability to alter fatty acids to meet their physiological and metabolic needs. Categories of fatty acids are the saturated fatty acids having no double bonds, monounsaturated fatty acids having one double bond, polyunsaturated fatty acids having two or more double bonds and the afore mentioned highly unsaturated, long-chain fatty acids having three or more double bonds. The latter category includes EPA and DHA, the primary fatty acids associated with human health. Levels of saturated fatty acids in fish tissues remain within a relatively narrow range regardless of dietary fat source whereas levels of monounsaturated fatty acids, such as oleic acid (C18:1), vary somewhat depending on dietary fat source. However, levels of polyunsaturated fatty acids in fish tissues, especially linoleic acid (C18:2n-6), are highly responsive to dietary level and can increase greatly when certain alternative lipid sources are present in the diet of fish. Olive, canola and peanut oils are rich sources of oleic acid, whereas corn, cottonseed, safflower, soy and sunflower oils are rich sources of linoleic acid. Substituting fish oil with oils high in linoleic acid significantly increases the level of linoleic acid in fillets and increases the ratio of n-6 to n-3 fatty acids, a negative outcome in terms of potential value to nutritional quality from the perspective of human health.

Fish oil is a by-product of fishmeal production and global production of both commodities is at maximum capacity with no further room to increase (Naylor *et al.* 2009). Alternative lipid sources are increasingly required in fish feeds to meet the demands of fish feed production associated with the growth of intensive aquaculture production and given predictions of continued growth of intensive aquaculture, the use of alternative lipids in fish feeds will grow. Over the recent decades, alternative oils, mainly from oilseeds, have replaced about half of the fish oil in fish feed formulations (Tacon & Metian 2008; Shepherd *et al.* 2017), leading to a decrease in EPA and DHA levels in fillets, especially salmon fillets (Nichols *et al.* 2014; Sprague *et al.*, 2016). The challenge for the aquaculture industry is to maintain healthful levels of EPA and DHA in farmed fish products as the percentage of alternative oil sources in fish feed formulations increases. There are several ways this can be accomplished. First, levels of EPA and DHA can be increased by feeding a 'finishing diet' during the final stages of grow-out prior to harvest. Finishing diets are those in which alternative (plant) oils in feeds used during the grow-out stage of production are replaced by fish oil or other high EPA/DHA oils in the final stages of growth before fish are harvested (Bell *et al.* 2003; Glencross *et al.* 2003d). The degree to which EPA and DHA levels can be increased depends upon dietary EPA and DHA levels as well as the duration of feeding. For the most part, fatty acid changes in fish fillets follow the 'dilution' hypothesis, meaning new fatty acids dilute those already deposited in tissues (Glencross *et al.* 2003d; Jobling 2003; Turchini *et al.* 2009). A second approach is to add high DHA ingredients, such as products from algae, to the diet. While this approach is not cost effective at today's prices, this may change in the future. A third approach may be to develop GMO oilseeds that produce EPA and/or DHA. A final approach may be to utilize selective breeding to improve the efficiency with which fish convert linolenic acid (C18:3n-3) in to EPA or DHA.

Putting perspective on these risks

The purpose of this review, in terms of a risk assessment, has been principally to identify the key risks in a qualitative sense (i.e. the consequences), but not necessarily in a quantitative manner (i.e. the likelihood). For a more comprehensive risk assessment, both components clearly need to be examined.

Consequences and likelihood

Quantifying the consequences and likelihood of certain risk factors is a challenge fraught with difficulties. Differences in perspective among different use sectors (e.g. ingredient producer cf. ingredient user), countries (e.g. EU cf. USA on GMO crops) and stakeholders (e.g. producers cf. insurers) all complicate the assessment. Because of this variability in perspectives we took approach among the authors of the paper, which covers geographical ranges (EU, USA, Scandinavia and Australia), stakeholders (nutritionists, toxicologists, immunologists and veterinarians) and we attempted to assess the consequences and likelihood of certain risk factors to both the fish and humans fed the fish on a basis independent from the various use sectors. From this approach, we tried to assess the risk relative to an industry standard (fishmeal). Only a generic approach (vegetable vs animal meals) was considered. A summary of those results is shown in Table 7.

From this assessment exercise what we noted was that compared to fishmeal, both vegetable and terrestrial animal derived feed raw materials offered a range of risk reduction opportunities and a small number of increased risk threats. While it was perceived that there were a greater number of potential risks with vegetable materials relative to the terrestrial animal derived feed raw materials, what such an assessment does not do is give any weighting to one risk over another. In this situation, some risks might be

Table 7 Perceived risk (less = −1; same = 0, more = 1) to fish and human consumer health when consuming fish fed diets based on the use of either vegetable or terrestrial animal derived feed raw materials relative to that from marine derived resources (fishmeals and oils)

Risk factor to fish	Vegetable	Animal
Variability		
Compositional	−0.5	0.0
Supply	−1.0	−1.0
Contaminants		
Antimicrobial residues	−0.5	1.0
Dioxins	−1.0	0.0
Other organochlorides	−0.3	0.0
Heavy metals	−1.0	−0.5
Pesticides	1.0	−0.5
Mycotoxins	1.0	−0.8
Antinutritional factors	1.0	−0.7
Zoonotic threats		
TSEs	−0.8	0.7
Viruses	−1.0	0.0
Parasites	−1.0	−0.3
Genetic modification	0.7	0.0
Risk factor to humans	**Vegetable**	**Animal**
Variability		
Compositional	−0.5	−0.3
Supply	−1.0	−0.8
Contaminants		
Antimicrobial residues	−0.5	0.5
Dioxins	−1.0	0.0
Other organochlorides	−0.3	0.0
Heavy metals	−1.0	−0.5
Pesticides	0.8	−0.5
Mycotoxins	1.0	−0.5
Antinutritional factors	0.7	−0.3
Zoonotic threats		
TSEs	−0.8	1.0
Viruses	−1.0	−0.3
Parasites	−1.0	−0.5
Genetic modification	0.3	0.0

considered of low consequence, but greater likelihood but of lower perceived overall risk than something of low likelihood but catastrophic consequences.

Prevention or cure?

In addition to the consideration of the consequences and likelihood of certain risks, the management of such risks also needs to consider the various options to their control, such as the prevention or remediation (cure).

Prevention, is arguably, always better than remediation. In the present case we refer to the prevention of risk entering the feed chain. A range of such strategies exist and are widespread across the sector but vary in their extent and detail. The most common strategy is the simple analysis of ingredients to assess both the type and extent of potential risk (Glencross *et al*. 2007a). For such analysis, there are certain standards that need to be considered to ensure reliability in the results and these standards and how they are defined vary from country to country (e.g. AOAC, EU, UKAS, etc.). However, exhaustive analytical testing is both cost and time prohibitive in most cases, so a degree of rationalization is applied subject to type the ingredients being assessed and potential risk factors of concern. One such point of value of the present review is to highlight those risks across the various ingredients in the aquaculture feed chain. Once data is obtained from such testing it is then used to inform about potential thresholds/exposure and the associated risk (Codex Alimentarius Commission 2017). For many of the major contaminant risks of concern, there are defined MRL in terms of what is allowable in an ingredient and a finished feed product (Codex Alimentarius Commission 2017).

Both ingredient and feed processing can also be used as a means of prevention of some risks. Ingredient processing is typically used to mitigate some ANF issues (e.g. cooking of soybean meal to denature protease inhibitors), while the conditions used in modern feed processing (e.g. extrusion) provide a degree of sterilization from microbes (Kelley & Walker 1999; Okelo *et al*. 2008). These are only two examples of the impacts of processing on risk mitigation, but there are a suite of other potential benefits that have been the subject of other reviews (Drew *et al*. 2007a).

There are also potential remedial actions to address some of the potential risks. A common one presently in use for many contaminants is the use of a 'withdrawal' period before fish enter the human food chain (Burridge *et al*. 2010). This strategy is consistent with the targeting of a specific MRL in the animal. Notably such withdrawal periods are usually not only time, but also temperature dependent (Abedini *et al*. 1998). However, a persistent concern is those MRLs for new compounds being developed that don't yet have a defined MRL. This clearly is an active area in certain sectors like pharmaceutical development, where clearance studies are routine to develop appropriate use guidelines before the drugs are made available for use (Abedini *et al*. 1998; Alderman & Hastings 1998). In addition to the use of MRL guidelines and their associated withdrawal criteria, another potential remedial action is the use of binders and adsorbents to bind toxic substances in either the raw materials or feed (Binder 2007). Several commercial products are available to mitigate the impacts of some risk factors like mycotoxins (Jouany 2007; Zhu *et al*. 2016). A third proposed remediation strategy has been to consider manipulation of the physiology of the animal to enhance the metabolic turnover and excretion of contaminants (Kan & Meijer 2007). In effect this approach is an extension of the MRL approach, but with the objective of decreasing the time taken to reach that MRL.

There are also risks about the movement of different raw materials produced in one country which may introduce residues from drugs/chemicals otherwise banned or restricted in another country (Kan & Meijer 2007). With an increasing degree of globalization in the international feed sector, a need to harmonize many of the feed associated regulations is emerging. Many of the companies now supplying the global aquaculture sector are multinationals and are trading across the world in both the developed and developing regions. Accordingly, the trade in aquaculture feed ingredients is also a global activity, with most major companies sourcing from across the globe. This globalization of the feed sector, like many of the other globalizations seen in the past decades is also likely to raise a suite of issues. There will be an increasing need for consistency in regulations and standards across the sector, irrespective of international boundaries. For there to be such standards to exist, there will clearly then need to be a degree of objectivity in those standards. However, there will also need to be some consideration of the rate of change in those standards as the international community seeks to obtain this consistency.

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