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Scientific and technical assistance concerning the survival, establishment and spread of *Batrachochytrium salamandrivorans* (Bsal) in the EU

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Abstract

A new fungus, *Batrachochytrium salamandrivorans* (Bsal), was identified in wild populations of salamanders in the Netherlands and Belgium, and in kept salamander populations in Germany and the United Kingdom. EFSA assessed the potential of Bsal to affect the health of wild and kept salamanders in the EU, the effectiveness and feasibility of a movement ban of traded salamanders, the validity, reliability and robustness of available diagnostic methods for Bsal detection, and possible alternative methods and feasible risk mitigation measures to ensure safe international and EU trade of salamanders and their products. Bsal was isolated and characterised in 2013 from a declining fire salamander (*Salamandra salamandra*) population in the Netherlands. Based on the available evidence, it is likely that Bsal is a sufficient cause for the death of *S. salamandra* both in the laboratory and in the wild. Despite small sample sizes, the available experimental evidence indicates that Bsal is associated with disease and death in individuals of 12 European and 3 Asian salamander species, and with high mortality rate outbreaks in kept salamanders. Bsal experimental infection was detected in individuals of at least one species pertaining to the families Salamandridae, Plethodontidae, Hynobiidae and Sirenidae. Movement bans constitute key risk mitigation measures to prevent pathogen spread into naïve areas and populations. The effectiveness of a movement ban is mainly dependent on the import volumes, possibility of Bsal to remain viable outside susceptible/tolerant species, and the capacity to limit illegal movements. Duplex real-time PCR can be used to detect Bsal DNA, but has not been fully validated. Quarantining salamanders, enacting legislation that requires testing of animals to demonstrate freedom from Bsal, before movement can take place, restricting salamander movements, tracking all traded species, hygienic procedures/biosecurity measures before and during movements, and increasing public awareness are relevant measures for ensuring safe intra-EU and international trade of salamanders.

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Keywords: *Batrachochytrium salamandrivorans*, salamanders, population decline, movement ban, diagnostic tests, safe trade

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Summary

A new fungus, *Batrachochytrium salamandrivorans* (Bsal), has been identified in wild populations of salamanders in the Netherlands and Belgium, and in kept populations in Germany and the UK.

In accordance with Article 31 of Regulation (EC) No 178/2002¹, the European Food Safety Authority (EFSA) was requested by the European Commission to compile and scrutinise available data on Bsal and to determine if Bsal is a disease with the potential to harm kept and wild salamanders in the European Union (EU).

Specifically, EFSA was asked to provide scientific and technical assistance concerning: (1) an assessment of the potential of Bsal to affect the health of wild and kept salamanders in the EU; (2) the effectiveness and feasibility of a movement ban (including intra-EU trade and introduction from non-EU countries) of traded salamanders, including both Asian and non-Asian species; (3) the validity, reliability and robustness of the available diagnostic methods for the detection of Bsal; and (4) possible alternative methods and feasible risk mitigation measures to ensure safe international and EU trade of salamanders and their products.

EFSA carried out an Extensive Literature Review on Bsal. Information from the papers selected as relevant and from additional literature identified by working group experts was used for a narrative description and assessment to address the four Terms of References (ToRs). To assess if a causal relationship between Bsal and mortality in salamanders exists, a critical appraisal of experimental infections of Caudata with Bsal was performed to evaluate the risk that results are biased. Official volumes of traded animals of the species of Caudata listed under CITES Appendices and/or Annexes of the EU Wildlife Trade Regulations, from 2005 to 2015, and relevant ancillary data (e.g. origin, purpose of the trade, importing and exporting countries, etc.), were analysed. The main factors which could potentially affect the feasibility and the effectiveness of a movement ban and the related uncertainty were qualitatively assessed by working group experts. An assessment of the validation of the duplex real-time polymerase chain reaction against the stages outlined in the OIE Manual of Diagnostic Tests for Aquatic Animals was performed. Available risk mitigation measures to ensure safe trade were assessed based on expert opinion, in terms of relevance and feasibility to ensure safe international and intra-EU trade.

Bsal, Kingdom Fungi, Phylum Chytridiomycota, Order Rhizophydiales, Family *incertae sedis*, genus *Batrachochytrium* is the second known chytrid fungus and shares many traits with the sister taxon, *Batrachochytrium dendrobatidis* (Bd). In culture, Bsal thrives best at 15°C. The zoospores actively swim in water, the fungus is dependent on water, and desiccation is fatal to all life stages.

Salamanders belong to the order Caudata of the class Amphibia. Nine families have been recognised, of which three, Plethodontidae, Proteidae and Salamandridae, have species in the EU. The family Salamandridae is the most widely distributed and rich in species family in Europe, represented here by 29 species, although the majority of species belonging to this family lives in Asia. According to reports under the Habitats Directive 2007–2012,² overall, nearly 40% of salamander species in Europe are considered to be in 'Unfavourable condition'. Over half of the European salamander species have been considered as 'Decreasing in abundance' by IUCN RedList experts.

Bsal was isolated and characterised in 2013 from a declining fire salamander (*S. salamandra*) population in Het Bunderbos, the Netherlands. Post-mortem examinations of deceased salamanders revealed intraepidermal organisms, intracellular structures consistent with colonial thalli of a new species of fungus were found in transmission electron microscopic examination of the skin lesions. There was no evidence for any other pathogen (Bd, viruses, Chlamydiaceae and bacteria).

In controlled experiments, healthy salamanders infected with 5,000 zoospores of Bsal, showed episodes of ataxia and died 12–18 d after exposure. Bsal was isolated from one infected animal, Bsal DNA was identified by polymerase chain reaction (PCR) in all animals, and histopathological examination showed focal epidermal ulcerations with high numbers of colonial Bsal thalli. Intraspecies transmission of Bsal was shown in a co-housing experiment, in which the two study salamanders died 22 and 27 days, respectively, after contact with the infected individual. Interspecies transmission of Bsal between susceptible species and from a presumed reservoir species to susceptible species were demonstrated in two infection experiments, resulting in increasing loads of Bsal DNA in skin swabs of all exposed

¹ Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety (OJ L 031, 1.2.2002, p. 1–24).

² Council Directive 92/43/EEC of 21 May 1992 on the conservation of natural habitats and of wild fauna and flora. Official Journal L 206, 22/7/1992 p. 7–50.

salamanders in consecutive samples. The host range of Bsal was estimated in infection experiments involving 161 animals from 35 species from the amphibian orders Anura, Caudata and Gymnophiona. Colonisation was limited to species of Caudata, while none of the other orders became infected. Forty-one of the 44 animals belonging to the families Salamandridae and Plethodontidae (Caudata) died rapidly after the infection. In an experimental infection exposing Caudata and Anura for 24 h to 10,000 Bsal zoospores, skin invasion ability of Bsal was shown to be limited to infect Caudata belonging to the families Salamandridae and Plethodontidae. The critical appraisal of these infection experiments showed that the overall risk of bias in relation to internal validity of the studies is considered to be low. However, most of the animals tested in the seven experiments came from the same field or captive source populations and are therefore expected to be correlated. In addition, the sample sizes used in the experiments are very low, ranging from one to eight animals per species. These numbers do not allow controlling for any potential confounders. Several experiments involving the most sensitive species fire salamanders were biologically highly significant, consistently resulting in up to 100% mortality in the exposed, versus 0% mortality in control treatments; on this basis, the biological relevance of the outcomes of the infection experiments is considered to indicate that Bsal is likely to be biologically associated with chytridiomycosis and death of many infected salamanders, with infection outcomes following exposure and mortality rates varying among species.

In observational studies, Bsal was demonstrated in clinical examinations of wild animals from declining populations, which died in captivity. It was also isolated and grown in culture from wild animals which died in captivity. Bsal was demonstrated in some salamanders declining populations, was not detected from some other and was detected in some salamander populations regarded as not in decline.

It was shown that exposure to $> 25^{\circ}\text{C}$ for at least 10 days, or a synergistic treatment with voriconazole, polymyxin E and temperature, eliminated the Bsal chytridiomycosis from wild and experimentally infected fire salamanders.

In addition to experimental infections of individuals of a range of amphibian species, several thousands of wild, captive and museum specimens were tested to identify the amphibian species susceptible to Bsal. The infection experiments involving salamanders showed that individuals of the species *Paramesotriton deloustali*, *Cynops pyrrhogaster* and *Cynops cyanurus* are susceptible to Bsal. Individuals of the families Salamandridae, Plethodontidae, Hynobiidae and Sirenidae are tolerant: they can carry the pathogen without showing any clinical sign. The only Caudata family from which none of the tested individuals developed clinical disease after infection is Ambystomatidae (which contains 36 species).

Bsal infections can be diagnosed by clinical examination, microscopy, PCR, isolation and culture. The clinical lesions linked to Bsal are characterised by marked skin ulcerations, but in general the clinical signs are variable and not pathognomonic. Microscopy and histological examinations of skin scrapings or skin sections from infected animals have demonstrated keratinocytes with eosinophilic necrosis and marginated nuclei at the periphery of the erosions. Transmission electron microscopy or immunohistochemistry can also be used demonstrate the intraepidermal structures. Considering the similarity of the lesions induced by Bd and Bsal, the basic histological examination as such cannot be used as a diagnostic test to discriminate between the two fungi. Several options for detecting Bsal in swabs, toeclips, skin sections, skin from moribund salamanders as well as environmental samples (water) using amplification of specific DNA sequences have been developed.

The commercial trade of the vast majority of the world's amphibian species is not regulated and they can therefore be freely shipped. It involves a wide range of captive-bred and wild-caught species, originating from multiple countries and involving an estimate of six million amphibians per year. Salamanders are traded at all life stages. The mode of transport is highly variable. Extrapolating from US government data on salamander trade, it is estimated that a total number of 620,000 individuals of Caudata have been imported into EU-28 between 2005 and 2015. Illegal movements are considered to take place in addition.

Movement bans constitute key risk mitigation measures to prevent (human-driven) pathogen spread into naïve areas and populations, particularly as management of invasive pathogens becomes difficult once they are established in wildlife populations. Therefore, import restrictions to limit pathogen introduction and early detection through surveillance of high-risk areas are priorities to control pathogen invasion. Major factors that could influence the effectiveness and the feasibility of a movement ban are considered to be: (i) the import volumes, (ii) the knowledge of the relevant species that need to be covered by the movement ban, (iii) the possibility of Bsal to remain viable outside

susceptible/tolerant species (e.g. fomites, travel boxes, etc.), and (iv) the illegal movement of susceptible animals.

A number of risk mitigation measures are considered potentially relevant to ensure safe imports into the EU and safe intra-EU movements as an alternative to a movement ban. However, further analysis of these measures is required before recommendations can be made regarding their feasibility and implementation for imports into-EU and for intra-EU movements.

Conclusions

Regarding the potential of Bsal to affect the health of wild and kept salamanders in the Union (ToR 1), it is concluded that, based on the currently available evidence, it is likely that Bsal is a sufficient cause for the death of at least one susceptible species, *S. salamandra*, both in the laboratory and in the wild. Despite small sample sizes, the experimental evidence to date further indicates that Bsal is associated with disease and death in 12 European and in 3 Asian salamander species, and is associated with high mortality rate outbreaks in kept salamanders. Experimental infection by Bsal was successful in individuals of at least one species pertaining to the families Salamandridae, Plethodontidae, Hynobiidae and Sirenidae.

Regarding the effectiveness and feasibility of a movement ban (including intra-EU trade and introduction from non-EU countries) of traded salamanders, including both Asian and non-Asian species (ToR 2), it is concluded that the effectiveness of a movement ban is mainly dependent on the import volumes, possibility of Bsal to remain viable outside susceptible/tolerant species (e.g. fomites, travel boxes, etc.) and the capacity to limit illegal movements. It is concluded that the feasibility of a movement ban mainly depends on the import volumes. Considering the complexity of the taxonomy as well as the lack of evidence related to all the species, a movement ban at the level of taxonomic order is likely to be both more effective and more feasible than a species-specific ban.

Regarding the validity, reliability and robustness of the available diagnostic methods for the detection of Bsal (ToR 3), from the assessment of the validation of duplex real-time PCR against the stages outlined in the OIE Manual of Diagnostic Tests for Aquatic Animals, it was concluded that the validation of the assay completed the first two validation stages, but not the third stage as foreseen in the OIE guidelines. Based on the estimates that current data lead to, it resulted that: (i) the test is not suitable for prevalence studies; (ii) the test could fail in detecting infected animals; and (iii) the test could still fit for a freedom from disease framework, although a safe approach would imply a considerably high sample size. These considerations are based and due to the statistically limited sample size used in the validation process and do not necessarily reflect the actual performance of the test.

Regarding possible alternative methods and feasible risk mitigation measures to ensure safe international and EU trade of salamanders and their products (ToR 4), it is concluded that (i) quarantining salamanders, (ii) enacting legislation that requires testing of the animals to demonstrate freedom from Bsal, before movement can take place, (iii) restricting salamander movements, (iv) tracking all traded species, (v) hygienic procedures/biosecurity measures before and during movements, and (vi) increasing public awareness, are relevant and feasible measures for ensuring safe intra-EU and international trade. Regarding quarantining salamanders, it is possible to estimate the sample size needed in order to assess, with a 95% confidence, if the consignment is free from Bsal, based on the number of animals included in the consignment and on the diagnostic sensitivity (DSe) of the test used. Assuming a worst-case scenario with a DSe equal to 0.5, (i) the size of the consignment cannot be smaller than 432, (ii) all animals should be tested and (iii) all test results should be negative. Different parameters and scenarios can be set according to the needs. In addition, animal by-products derived from salamanders that have been heat treated at 25°C for at least 10 days or desiccated are not considered relevant for the spread of Bsal to the salamander populations in the EU.

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1. Introduction

1.1. Background and Terms of Reference as provided by the European Commission

Batrachochytrium salamandrivorans (Bsal) was identified by scientists as recently as in 2013. Over the last couple of years, Bsal occurred at least in certain parts of Europe either in wild population of salamanders and newts (e.g. the Netherlands, Belgium in several locations) or in kept population (e.g. Germany, the UK) or possibly in both populations. There is no data from other European Union (EU) countries but similar cases either in wild or in kept salamanders cannot be excluded. In certain areas (e.g. the Netherlands), this fungus is said to have devastated local fire salamander populations. In other places, the fungus is apparently present in susceptible species, but without increased mortalities. Many Asian salamander species seem to be immune or tolerant to this pathogen to various extents. Many European species seem to be susceptible.

This situation and state of knowledge is patchy, fragmented and is expected to change as more knowledge about this emerging pathogen and especially surveillance data becomes available, continually and gradually. Overall, scientific data on Bsal is still scarce with significant gaps. Currently, the disease is not listed under OIE standards or in the EU rules.

A few affected countries, or those which anticipate that Bsal could affect them, adopted diverse control policies or consider various possible measures against the disease to cope with its feared short- and long-term consequences in wild animals and in kept salamanders and their trade. One of such examples is an import ban of certain salamander species introduced by the USA, where Bsal is either absent or not yet detected. Other measures are of non-legislative nature, such as rising of awareness among stakeholders on risks, guidelines for improved biosecurity or survey salamander populations, changes thereof, with the emphasis on increased mortalities and/or occurrence of Bsal. To date, these have been done under environmental policies.

Some actors have recently called for, *inter alia*, EU animal health policy and legislative measures to be adopted, in particular an immediate ban on import of many species of salamanders from Asia into the EU. It has been shown by phylogenetic analysis that the fungus indeed originates in certain parts of Asia. Therefore, it has been speculated by some that trade in Asian salamanders may play a role in its spread into and within the EU, although there is no proof that this pathogen entered the EU via this route, and if so, when and under what circumstances. In general, details are missing on its actual spread into or in the EU or between kept and wild animals.

The Commission therefore needs a quick but comprehensive compilation, scrutiny of available data and assessment to determine if Bsal is a disease with the potential to harm kept and wild salamanders in the Union and various risk factors associated with:

- imports of Asian salamanders into the EU and their trade within the EU;
- movements of European salamanders (both caught from the wild or kept ones) within the EU; and
- imports and movements of animal by-products obtained from Asian and European salamanders (both caught from the wild or kept ones).

Such assessment would be essential for the consideration of potential safeguard measures in relation to imports from Asia or for movements from infected to non-infected EU areas.

In the past, the European Food Safety Authority (EFSA) has produced scientific opinions dealing with various aspects of emerging pathogens, including those where environmental aspects or wild animals play increased role or are affected (e.g. on small hive beetle). Therefore, a similar opinion is necessary to understand better possible scenarios for the evolution of this new disease, the current epidemiological situation, the experience gained so far from the implementation of the various control policies and possible alternative methods to diminish negative effects on wild salamander populations and to ensure safe trade of kept animals and their products. Identification of gaps and uncertainties is also very important for this emerging disease.

Furthermore, EFSA has already been made aware of the adoption and publication of the Regulation on transmissible animal diseases (Animal Health Law), hereinafter referred to as AHL. As Bsal is not included in the list of diseases in Annex II to the AHL (or on the list of any other existing EU animal health legislation), environmental actors asked the Commission to place Bsal onto that list. Therefore, the review of this list will be necessary in accordance with a set of criteria provided for in the AHL before it comes into force, taking into account the transitional periods envisaged for its application

(5 years starting from April 2016). Hence, the Commission needs scientific advice for the assessment of the significance of Bsal within the framework of this already known listing and categorisation according to the AHL, in the same manner it was requested previously, for another two groups of diseases (Ref. SANTE G2/BL/lp (2015) 4940871, SANTE G3/LPA/lp (2016) 3154863, respectively).

The criteria, provided for in Article 7 and 8 and Annex IV of the AHL shall be used as a basis for this analytical assessment. The risk manager needs a scientific advice in order to:

- 1) assess if Bsal causes disease for which control measures at the EU level are justified;
- 2) proceed with the profiling of the disease in view to its categorisation; and
- 3) assign listed species to Bsal identified as eligible for EU intervention.

The Commission have identified the main issues for which concrete elements of science may provide good basis for formulating policies and/or adapt current approach. These are as follows:

- provisions for safe trade (entry into the Union and trade within the Union) with Asian and European salamanders and animal by-products obtained therefrom;
- identifying links between groups of salamanders in trade (i.e. in consignments being moved or in shops etc.) and kept ones (i.e. stationary, whether for hobby or else) and salamanders in wild (i.e. in their natural habitat), and possible routes and risks of spreading Bsal between the specimens belonging to the above three groups and locations;
- effects of the respective infection of salamanders with Bsal, including aspects stemming from different susceptibility of various species to Bsal;
- measures to monitor occurrence of Bsal in those groups and mitigate mortality due to Bsal, whether regulatory measures or non-regulatory ones.

1.1.1. Terms of Reference

I. Scientific and technical assistance in accordance with Article 31 of Regulation (EC) No 178/2002

In view of the above, in accordance with Article 31 of Regulation (EC) No 178/2002, the Commission asks EFSA to provide scientific and technical assistance concerning:

- 1) assessment of the potential of Bsal to affect the health of wild and kept salamanders in the Union;
- 2) effectiveness and feasibility of a movement (including intra-EU trade and introduction from non-EU countries) ban of traded salamanders, including both Asian and non-Asian species;
- 3) the validity, reliability and robustness of the available diagnostic methods for the detection of Bsal;
- 4) possible alternative methods and feasible risk mitigation measures to ensure safe international and EU trade of salamanders and their products.

II. Scientific opinion in accordance with Article 29 of Regulation (EC) No 178/2002

In accordance with Article 29 of Regulation (EC) No 178/2002, the Commission asks EFSA to provide a scientific opinion on the following:

1) As regards susceptibility, morbidity and mortality, assess:

- a) susceptibility and morbidity of various Asian and European salamanders to Bsal;
- b) nature of Bsal as facultative or not pathogen of European salamanders;
- c) if there are species of salamanders carrying Bsal without clinical symptoms and/or clinical and serological evidence and if so, which ones;
- d) mortality rates of native European salamander species due to Bsal;
- e) role of other factors (e.g. habitat degradation, etc.) in increased mortalities associated with Bsal.

2) As regards presence, absence, surveillance and eradication, assess:

- a) the risk of survival and establishment of Bsal in the environment in the EU under various meteorological conditions;
- b) possible identification of various areas (e.g. countries, zones, territories etc.) which may be considered infected with Bsal or free from it;

- c) definition of requirements for reliable detection of Bsal in the wild in affected areas or exclusion of its presence;
- d) suitability of surveillance methods to ensure reliable and robust demonstration of presence or absence of Bsal.

3) As regards spread of Bsal in and from infected areas or via infected animals or fomites, assess:

- a) the risk of survival, spread and establishment of Bsal within already infected areas and spread from infected areas into other parts of the EU under various scenarios:
 - i) by natural movements of live salamanders taking into account especially relevant geographical, hydrographical and meteorological conditions;
 - ii) by movements of traded live salamanders and their traded products, body parts etc. from infected areas, both under identified risk mitigation measures or without;
- b) risk mitigating factors that could potentially be effective in ensuring safe international or intra-EU trade of live salamanders (both captured in the wild and bred) and their products and by-products as regards the transmission of Bsal including diagnosis and potential treatment(s);
- c) the role of live, silent carriers of Bsal in spreading it as vectors and those of fomites (e.g. waste water, animal by-products, feed etc.) and risk mitigating measures concerning those;
- d) the possible routes of spread between kept salamanders, originating from international trade and the autochthonous salamanders living in wild, i.e. their natural habitat.

4) As regards on-site protection from Bsal, assess:

- a) potential and feasible risk mitigating factors and methods in kept salamanders;
- b) risk mitigating factors and methods for salamanders in their natural habitat.

5) Listing and categorisation of Bsal in the framework of the Animal Health Law:

- a) Assess, following the criteria laid down in Article 7 of the AHL, its eligibility of being listed for Union intervention as laid down in Article 5(3) of the AHL;
- b) If found eligible to be listed for Union intervention, provide:
 - i) an assessment of its compliance with each of the criteria in Annex IV to the AHL for the purpose of categorisation of diseases in accordance with Article 9 of the AHL;
 - ii) a list of animal species that should be considered candidates for listing in accordance with Article 8 of the AHL.

1.2. Interpretation of the Terms of Reference

This Scientific Report aims at addressing the four Terms of References (ToRs) relevant to the Scientific and technical assistance in accordance with Article 31 of Regulation (EC) No 178/2002 (see point I above). It aims at addressing the need of the European Commission to have a quick but comprehensive compilation, scrutiny of available data and assessment to determine if Bsal is a pathogen with the potential to harm kept and wild salamanders in the Union and various risk factors associated with: (i) imports of Asian salamanders into the EU and their trade within the EU; (ii) movements of European salamanders (both caught from the wild or kept ones) within the EU, and (iii) imports and movements of animal by-products obtained from Asian and European salamanders (both caught from the wild or kept ones).

In this report, the potential of Bsal to affect the health of wild and kept salamanders in the EU (ToR 1), the methods to identify the fungus (ToR 3), and the effectiveness and feasibility of a movement/trade ban (ToR 2) were assessed before addressing the possible alternative methods and feasible risk mitigation measures to ensure safe trade (ToR 4).

Regarding the first term of reference related to the potential of Bsal to affect the health of wild and kept salamanders in the EU, EFSA assessed, based on the available scientific evidence, if the pathogen is associated with the death of the affected animals. Further, it was assessed which amphibian species or families may play a role in the introduction and spread of Bsal in the EU (ToR 1).

Regarding the third term of reference related to the validity, reliability and robustness of the available diagnostic methods for the detection of Bsal, EFSA identified available methods and assessed the quality

of the test performance evaluations reported in scientific literature. The test parameters relevant for understanding the epidemiology of the disease, i.e. sensitivity and specificity, are reported (ToR 3).

Regarding the second term of reference, this assessment provides an overview of the trade of salamanders and some epidemiological and technical considerations on the effect that a movement ban (including intra-EU trade and introduction from non-EU countries) of traded salamanders (both Asian and non-Asian species) may have on the introduction and spread of Bsal in the EU (ToR 2). The possible alternative methods and risk mitigation measures to ensure safe international and EU trade of salamanders and their products, were also described and assessed (ToR 4).

A Scientific opinion addressing the additional ToRs sent by the requestors in accordance with Article 29 of Regulation (EC) No 178/2002 (see point II above) will be developed depending on the outcome of this assessment.

2. Data and methodologies

2.1. Data

2.1.1. Data from literature

Information from the papers selected as relevant from the Extensive Literature Review (ELR) described in Section 2.2.1 and from additional literature identified by the working group experts was used for a narrative description and assessment to address ToRs 1, 2, 3 and 4 (see relevant sections in the Assessment chapter).

2.1.2. Data from Member States and databases

2.1.2.1. Population data

Data on the size and distribution of salamander populations in the EU were requested from the EFSA Animal Health and Welfare (AHAW) Network Representatives.³ Eleven countries provided information; however, only few Member States (MSs) (the Netherlands, Belgium, Germany, Croatia, Spain) have detailed data of salamander populations at national level. In some cases, data are collected in national databases (the Netherlands and Belgium), on the basis of monitoring programs (Belgium and Croatia), or for a regional atlas on the density of amphibians (Spain). The results show that the collection of data on salamander populations' size and distribution is fragmented and not harmonised across the MSs.

In addition, two websites identified by the working group experts, Amphibiaweb⁴ and the IUCN Red List,⁵ were used to gather data on salamanders. The 'IUCN Red List of Threatened Species' database provides taxonomic, conservation status and distribution information on plants, fungi and animals that have been globally evaluated using the IUCN Red List Categories and Criteria. Similarly, the 'AmphibiaWeb' website contains information on amphibian biology and conservation.

2.1.2.2. Trade data

The Convention on International Trade in Endangered Species of Wild Fauna and Flora⁶ regulates international trade in more than 35,000 animal and plant species. Contracting parties provide reports on a yearly basis to the competent CITES Secretariat, including full details of all export and import permits and certificates issued during the previous year. Worldwide, more than 850,000 records of trade in CITES-listed species of wildlife are reported annually.

At the EU level, according to Regulation (EC) No 338/97⁷, the CITES convention has been implemented by the EU Wildlife Trade Regulations which are directly applicable in the MSs. Both CITES and the EU Wildlife Trade Regulations cover trade in all specimens, whether alive or dead, including parts and derivatives, from animal and plant species listed in the Appendices and Annexes, respectively. Therefore, the term 'trade' encompasses not only trade in a commercial sense but also, for example, imports and (re)-exports for personal use.

³ <https://www.efsa.europa.eu/en/animal-health-and-welfare/networks>

⁴ <http://www.amphibiaweb.org>

⁵ <http://www.iucnredlist.org>

⁶ Convention on International Trade in Endangered Species of Wild Fauna and Flora, signed at Washington, D.C., on 3 March 1973 Amended at Bonn, on 22 June 1979 Amended at Gaborone, on 30 April 1983. <https://cites.org/eng/disc/text.php>

⁷ Council Regulation (EC) No 338/97 of 9 December 1996 on the protection of species of wild fauna and flora by regulating trade therein. OJ L 61, 3.3.1997, p. 1–69.

Similarly, the Trade Control and Expert System (TRACES)⁸ collects data on intra-EU trade and importation of animals, semen and embryos, food, feed and plants. However, currently in TRACES data on amphibians are grouped at class level, without specifying details per 'order' (e.g. Caudata), 'species' or 'genus'. This data does not have sufficient granularity for the analyses required for this report.

Therefore, for this report, official volumes of traded animals, (both direct and indirect imports) from 2005 to 2015, and relevant ancillary data (e.g. origin, purpose of the trade, importing and exporting countries, etc.), were provided by UNEP-WCMC⁹ for the species of Caudata listed under CITES Appendices and/or Annexes of the EU Wildlife Trade Regulations (see Appendix A – for the full list of Caudata species currently listed in the CITES Appendices and/or Annexes of EU Wildlife Trade Regulations). UNEP-WCMC was contacted for clarifications regarding the interpretation of the data reported in the CITES Trade Database. These clarifications were considered when analysing and describing the data (see Appendix G).

Additional trade data of Caudata (from 2005 to 2014) presented in the UNEP-WCMC report (2016) were also taken into account.

2.2. Methodologies

2.2.1. Extensive Literature Review

An ELR¹⁰ was carried out in order to identify scientific evidence on Bsal in wild and kept salamanders and newts in the EU.

The search aimed at identifying any peer-reviewed and grey literature containing information on Bsal. No restriction on the host population was applied, in order to identify all studies concerning the fungus. The search was carried out in the information resources listed in Appendix B, and no language, date or document type restrictions were applied in the search strings. The search strings were adapted to the configuration of each information resource. Full details of the search protocol and strategies are provided in Appendix B.

The number of results retrieved from each information source was recorded. The output of the searches, i.e. records retrieved from bibliographic databases and grey literature was exported to EndNote x7 together with the relevant metadata (e.g. title, authors, abstract).

The search yielded a total of 528 records. Duplicate records were compared and removed when two or more records were identical (i.e. author/s, title, journal, pages and doi number). After de-duplication, the search resulted into 311 records. A first screening of all titles and abstracts was performed in order to remove additional duplicates (if any) and to identify the literature pertaining to Bsal, leading to 67 records. The full text of these publications was assessed for being peer-reviewed primary studies or grey literature relevant for Bsal. A total number of 33 records (30 publications and 3 supplementing materials) relevant to the search question were identified. For each paper, it was indicated for which ToR (one or more) it contained relevant information. Full details of the numbers of the records that resulted from each step of the ELR are reported in Appendix B.

The reference lists of relevant review articles and key reports were checked for further relevant articles, and working group experts were invited to propose any additional relevant publications they were aware of.

As part of addressing ToR 1, a range of eligibility criteria were established in order to identify studies that allow assessing if a causal relationship between Bsal and mortality in salamanders exists. These were:

- target population salamanders or newts;
- experimental study with a control group;
- description of route of exposure;
- description of levels/doses used for the infection;
- description of duration of the exposure and the follow-up;
- description of temperature;
- description of observed mortality or of population decline or other adverse effect on health;
- description of the place the experiment was carried out;
- paper written in English (at least the abstract).

⁸ http://ec.europa.eu/food/animals/traces_en

⁹ www.unep-wcmc.org

¹⁰ The literature review was based on the principles of the EFSA Guidance on systematic review methodology (EFSA, 2010).

The 15 papers relevant for ToR 1 were screened for their eligibility against these criteria. Two publications that reported experimental studies fulfilling all eligibility criteria were identified (Martel et al., 2013, 2014). These were included in the critical appraisal described in Section 3.3.1.2.

2.2.2. Data extraction

Information from the papers selected as relevant for ToRs 1, 2, 3 and 4 was used for a narrative and descriptive assessment of these ToRs (see relevant sections in the Assessment chapter).

For ToR 1, data from experimental infection studies was extracted and used to critically appraise the study design.

2.2.3. Assessment of diagnostic tests

Regarding ToR 3, related to the validity, reliability and robustness of the available diagnostic methods for the detection of Bsal, EFSA screened all relevant scientific papers resulting from the ELR. A brief overview of the evolution of the diagnostic methods is given, followed by a technical description of the diagnostic test most used at present, i.e. the duplex real-time polymerase chain reaction. For the latter, the performance indicators are reported, as assessed in literature (Bloom et al., 2013). In addition, EFSA estimated the diagnostic sensitivity and the diagnostic specificity using the open source software 'R' (R Core Team, 2016), based on the available data in order to estimate the uncertainty around those key parameters. Last, an assessment of the validation process as described in the OIE Manual of Diagnostic Tests for Aquatic Animals was performed (OIE, 2016).

2.2.4. Assessment of the feasibility and effectiveness of a movement ban of traded salamanders

Regarding ToR 2, the experts were asked to extract from the available knowledge a list containing the main factors which could potentially affect the feasibility and the effectiveness of a movement ban. For each factor, a qualitative assessment of the related uncertainty was performed. The impact of each factor on the feasibility and the effectiveness of a movement ban is proposed in relation to the possible scenarios that the currently available knowledge suggests.

2.2.5. Assessment of alternative risk mitigation measures to ensure safe international and EU trade of salamanders and their products

In relation to ToR 4, a review of the available risk mitigation measures to ensure safe trade listed in Grant et al. (2016a) was performed. Each option was described and assessed, based on expert opinion, in terms of relevance and feasibility to ensure safe international and intra-EU trade. Additional options were proposed by the experts and assessed.

2.2.6. Critical appraisal

In the context of assessing if an association between Bsal and disease/mortality in salamanders exists (ToR 1), the two experimental infection studies identified by the ELR were critically appraised to evaluate the risk that results are biased. To this end, a Critical Appraisal Tool for Risk of Bias in experimental and observational studies, developed by EFSA, based on the tool of the NTP-OHAT¹¹ (NTP, 2015), was applied. Data on the experimental setting and the outcome assessment were extracted and assessed by a multidisciplinary team, composed of a domain expert and two methodologists, for potential risks of bias. Specifically, it was assessed if the allocation of animals to study groups had been randomised, and if personnel had been blinded; if confounding factors potentially influencing the results were controlled in the experimental setting; if outcome data is complete without attrition or exclusion from the analysis or if exclusion of individuals from the analysis had been done only if justified and without introduction of a bias. Further, it was assessed if exposure characterisation and outcome assessment were appropriate and if all outcome measures had been reported. Finally, it was assessed if a biologically relevant effect had been identified, if the sample size, the statistical methods used to summarise data, and the method used to treat missing data were appropriate. The corresponding author of the experimental infection studies was contacted for clarifications regarding exposure and outcome assessment details that had not

¹¹ National Toxicology Program – Office of Health Assessment and Translation.

been reported in the scientific publications. These clarifications were considered in the critical appraisal in addition to the information provided in the publications.

3. Assessment

3.1. Chytridiomycetes infecting amphibians

Batrachochytrium salamandrivorans (Bsal), Kingdom Fungi, Phylum Chytridiomycota, Order Rhizophydiales, Family *incertae sedis*, genus *Batrachochytrium* (see Figure 1), was first described in 2013 (Martel et al., 2013).

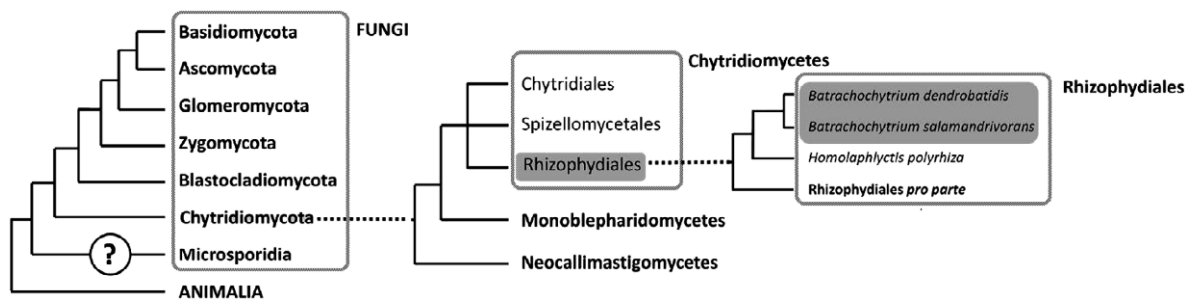


Figure 1: Illustration of phylogenetic position of *B. salamandrivorans* on combined cladograms based on Van Rooij et al. (2015). Branch lengths are not proportional to genetic distances

The scientific description of Bsal was based on a culture obtained from diseased individuals of fire salamander *Salamandra salamandra*. It is the second fungus species known from the genus, and shares many traits with the sister taxon, *Batrachochytrium dendrobatidis* (Bd; Longcore et al., 1999), with which it forms a clade.

The life cycle of both *Batrachochytrium* species contains aquatic motile zoospores and either colonial or monocentric thalli that produce sporangia. Sporangia of Bsal reach a size of 15.7–50.3 μm (average, 27.9 μm); in comparison, Bd sporangia grow up to 40 μm (Longcore et al., 1999). The asexually produced zoospores of Bsal are released by discharge papillae (Martel et al., 2013; Van Rooij et al., 2015).

The two *Batrachochytrium* species differ in several traits observed in culture. While Bsal thrives best at 15°C (Martel et al., 2013), Bd has its temperature optimum at 22°C (Longcore et al., 1999). In Bsal, the germ tubes arising from encysted zoospores form tubular extension from which new sporangia grow; Bsal often grows in form of colonial thalli (Van Rooij et al., 2015).

As Bd has been intensively studied since its detection in 1998 (Berger et al., 1998), the amount of available data on this species is much greater than the data available on Bsal. While the two species have a genetic distance¹² large enough to warrant a description of a separate species, they are still close enough to allow making some generalisations regarding basic biological aspects for both species of the genus.

Zoospores actively swim in water, in case of Bd the zoospores use chemical cues to locate amphibian skin (Moss et al., 2008; Van Rooij et al., 2015) and the fungus can remain viable in water for up to 3 weeks (Johnson and Speare, 2003). These fungi are dependent on water and desiccation is fatal to all life stages (Johnson et al., 2003; Van Rooij et al., 2015). Under laboratory conditions, both species can attach and grow on keratin containing substrates, although keratin is not an essential nutrient (Van Rooij et al., 2015).

The origin of Bsal cannot be explained as a variation or recombination of known Bd lineages as Bsal has its own long evolutionary history (Martel et al., 2014). In Europe, Bsal emerged in one location in the Netherlands, in a *S. salamandra* population experiencing a rapid decline (Spitzen-van der Sluijs et al., 2013). The Netherlands is believed to be the initial point of entry of Bsal to wild populations of European salamanders (Martel et al., 2014). The known area of distribution of Bsal in wild populations has expanded to Belgium and Germany since the first detection (Martel et al., 2014; Spitzen-van der Sluijs et al., 2016), but appears to be surrounded by yet uninvaded areas. The present known distribution of Bsal measured as minimum convex polygon encompassing the outermost out of 14 positive sites in the

¹² 3.47–4.47% sequence difference for 1,513 base pairs of 18S + 28S rRNA (Martel et al., 2013).

Netherlands, Belgium and Germany covers approximately 10,000 km² (see Appendix E). This area is in the proximity of 34 sites where Bsal was not detected (Spitzen-van der Sluijs et al., 2016). The fungus has also been detected in imported salamanders and in captive collections of salamanders in the UK (Cunningham et al., 2015) and in Germany (Sabino-Pinto et al., 2015).

Several studies have assessed the potential occurrence of the pathogen in other geographic locations. Bsal has not been identified in tested individuals from declining populations of the aquatic salamander *Cryptobranchus alleganiensis alleganiensis* (Bales et al., 2015) or in Appalachian Plethodon salamanders (Muletz et al., 2014) tested in the US, nor in selected fire salamander populations near Salzburg, Austria (Gimeno et al., 2015) or in 509 specimens representing 17 species of salamanders from the Swiss Alps, the Peruvian Andes and the Smoky Mountains in the United States (Parrott et al., 2016).

3.2. Salamanders in EU

3.2.1. Taxonomy

The taxonomical class Amphibia contains three extant orders: Anura (frogs and toads: 6,678 species), Gymnophiona (caecilians: 205 species) and Caudata (salamanders: 703 species). The amphibians in Europe belong to two distinct taxonomical orders: Anura and Caudata.

The salamanders are separated into nine recognised families (Frost, 2016, accessed on 18/01/2017; see Table 1).

Table 1: The species richness of salamanders by families and their geographic distribution, according to Frost (2016) and amphibiaweb^(a)

Family	Number of species	Distribution in the wild
Ambystomatidae	37	North America
Amphiumidae	3	North America
Cryptobranchidae	4	North America, Asia
Hynobiidae	67	Asia
Plethodontidae	460	Asia (Korea), Europe (Italy, France), North and South America
Proteidae	8	Europe, North America
Rhyacotritonidae	4	North America
Salamandridae	116	Asia, Europe, North America
Sirenidae	4	North America

(a): <http://www.amphibiaweb.org/lists/index.shtml>.

Out of the nine families in the order Caudata (see Table 1), three have species present in EU: Plethodontidae, Proteidae and Salamandridae (Temple and Cox, 2009).

The family Proteidae contains a single European species, the olm (*Proteus anguinus*), a fully aquatic salamander living in caves. The family Plethodontidae, lungless salamanders, is represented in Europe by eight species out of the 460 described at global level. The family Salamandridae is the most widely distributed and rich in species family in Europe, represented here by 29 species (Temple and Cox, 2009; Sillero et al., 2014), although the majority of species belonging to this family lives in Asia. Representatives of one group within the family Salamandridae (subfamily Pleurodelinae) are called 'newts'. Newts are more dependent on aquatic habitats than many other salamanders and spend a significant proportion of the year in water even as adults. For taxonomic consistency, it has to be noted that in this report the use of the term 'salamanders' is inclusive of 'newts'.

3.2.2. Distribution and abundance

The preferred habitats, life histories and behaviours of individual species of European salamanders vary greatly: from fully aquatic species, unable to survive on dry land, through species alternating between aquatic and terrestrial habitats, to species that can be considered fully terrestrial and not dependent on water for reproduction. These differences explain the broad diffusion of salamanders across Europe (see Appendix C), although the distribution of individual species varies significantly.

The family Proteidae, represented by *P. anguinus*, is restricted to caves of the Dinaric Alps (Italy, Slovenia, Croatia, Bosnia and Herzegovina; introduced to France).

The distribution of family Plethodontidae in Europe is also limited, with all *Speleomantes* species present only in Italy, with the exception of one species reaching south-eastern France.

Distribution of the members belonging to the family Salamandridae covers most of the continent, from the Mediterranean region to southern parts of Scandinavia (Sillero et al., 2014). The three species with the largest distributions (area of extent) are *Triturus cristatus*, *Lissotriton vulgaris* and *S. salamandra* (see Appendix C). The EU countries with the highest biodiversity (number of species) of salamanders are Italy, France and Spain.

The EFSA AHAW Network was consulted to check if additional data were available on salamander population sizes and distributions in MSs. While some data are available, collection of data is not harmonised across countries, therefore, the information is fragmented and not homogeneous, limiting its use for risk assessment purposes.

3.2.3. Conservation status and trends

According to reports under the Habitats Directive Period 2007–2012,² overall, nearly 40% of salamander species in Europe are considered to be in 'Unfavourable condition'. In the IUCN Red list, 11% of European salamanders are considered 'Endangered' or 'Critically Endangered', 21% are 'Vulnerable' and 21% are 'Near Threatened' (Appendix C). However, the conservation status of several species has not yet been assessed.

Regarding population trends, over half of the European salamander species have been considered as 'Decreasing in abundance' by IUCN RedList experts before the potential impact of Bsal was recognised. The most significant recognised threats to European amphibians are habitat loss, fragmentation, degradation and invasive alien species (Temple and Cox, 2009). The potential impact of novel pathogens, except Bd, on salamanders has not yet been included in the existing conservation assessment schemes.

European countries and EU Member States are signatories to several conventions aimed at biodiversity conservation that are relevant to amphibians. They include: (i) the 1979 Bern Convention on the Conservation of European Wildlife and Natural Habitats,¹³ (ii) the 1991 Convention on the Protection of the Alps,¹⁴ and (iii) the 1992 United Nations Convention on Biological Diversity.¹⁵ All countries and many lower administrative units (regions, provinces, etc.) have various forms of legislation on species protection (Temple and Cox, 2009).

3.3. Potential of Bsal to affect the health of wild and kept salamanders (ToR 1)

In order to characterise the potential of Bsal to affect the health of wild and kept salamanders, currently available scientific evidence has been assessed to establish if the pathogen is a sufficient cause for death of susceptible species. In a second step, it has been assessed if Bsal is the only explanatory variable of the population decline observed in wild and captive salamander populations.

3.3.1. Is the pathogen a sufficient cause for the death of salamanders?

3.3.1.1. First isolation and characterisation of Bsal

Martel et al. (2013) isolated and characterised a unique chytrid fungus, *Batrachochytrium salamandrivorans* sp. nov. (Bsal), from a declining fire salamander (*S. salamandra*) population in Het Bunderbos, the Netherlands.

For details on the decline of this population, see Spitzen-van der Sluijs et al. (2013).

Some (N = 39) of the remaining animals in the population were brought into captivity in 2012 as part of an *ex situ* conservation programme. The programme was compromised when 49% of the captive salamanders died (November–December 2012). The affected fire salamanders died within 7 days of having been taken into captivity, after a short episode of anorexia, apathy and ataxia. Post-mortem examinations of six of these deceased salamanders revealed intraepidermal organisms that did stain with immunohistochemistry, as developed by Hyatt et al. (2007), to detect Bd. Transmission electron microscopic examination of the skin lesions confirmed the presence of intracellular structures consistent with colonial thalli of a new species of fungus. There was no evidence for any other

¹³ <http://www.coe.int/en/web/bern-convention>

¹⁴ Council Decision 96/191/EC of 26 February 1996 concerning the conclusion of the Convention on the Protection of the Alps (Alpine Convention). Official Journal L 61, 12.3.1996, p. 31–31.

¹⁵ <https://www.cbd.int/doc/legal/cbd-en.pdf>

pathogen (Bd, viruses, Chlamydiaceae, bacteria). A new fungus, Bsal, was isolated from the skin of some of the fire salamanders.

3.3.1.2. Infection experiments

The ELR identified two scientific publications describing experimental infections of amphibians with Bsal (Martel et al., 2013, 2014). These included seven experiments.

In one experiment, five individually housed fire salamanders (*S. salamandra*) were infected by dripping 1 mL of filtered pond water containing 5,000 zoospores of Bsal on each animal. After 24 h, each animal was moved to a new container. The animals were followed up by clinical examination and weekly skin swab collection until 3 weeks after exposure. All five animals showed a 1–2 days episode of ataxia and died 12–18 days after exposure. The pathogen was isolated from one animal; Bsal DNA was identified by polymerase chain reaction (PCR) in all animals, and histopathological examination showed focal epidermal ulcerations with high numbers of colonial Bsal thalli. No clinical symptoms, lesions or death were observed in the five uninfected control animals that had been housed individually under the same conditions as the study animals (Experiment 1; Martel et al., 2013).

Intraspecies transmission of Bsal was assessed by cohousing two healthy fire salamanders with an infected individual for 2 days. The two study animals died 22 and 27 days, respectively, after contact with the infected individual; Bsal presence was shown in their epidermal layers by PCR, histology and immunohistochemistry (Experiment 2; Martel et al., 2013).

Five midwife toads (*Alytes obstetricans*) were exposed to 5,000 zoospores each and kept and observed under the same conditions described for Experiment 1. No animal showed any sign of disease or colonisation, as assessed by immunohistochemistry and PCR (Experiment 3; Martel et al., 2013).

To estimate the host range of Bsal, a total of 161 animals from 35 species from the amphibian orders Anura (48 animals from 10 species), Caudata (112 animals from 24 species) and Gymnophiona (one animal from one species) were individually exposed to 5,000 Bsal zoospores each and kept under the same conditions as described for Experiment 1. Most of the study animals were bred in captivity and most had been derived from the same source population. Daily monitoring for clinical signs and weekly swabbing of skin for quantitative PCR analysis were carried out; histopathology was carried out on animals that died. Animals were observed for at least 4 weeks or until they died or until they had three negative PCR results in three consecutive weeks. Morbidity, mortality and average days to mortality were reported. Colonisation was limited to species of Caudata, while no anurans or the caecilian became infected. Forty-one of the 44 animals belonging to the families Salamandridae and Plethodontidae (Caudata) died rapidly after the infection. These experiments were used to classify species into one of four categories of susceptibility, namely resistant, tolerant, susceptible and lethal (see Section 3.3.3) (Experiment 4; Martel et al., 2014).

The ability of Bsal to invade the skin of amphibians was assessed by exposing 14 animals of seven anuran species and 20 animals of 10 Caudata species (two animals per species) for 24 h to 10,000 zoospores per animal, after which they were immediately euthanised. The abdominal skin was investigated immunohistochemically for Bsal. Bsal was detected in the skin of some of the infected Caudata (belonging to the families Salamandridae and Plethodontidae), but not in the skin of any of the infected Anuran (Experiment 5; Martel et al., 2014).

To assess interspecies transmission of Bsal between susceptible species, four *Ichthyosaura alpestris* and two *Pleurodeles waltl* were co-housed 1:1 for 8 h with six infected *S. salamandra*, whose mean log₁₀ genomic load of Bsal was 1.74 ± 0.12 per swab. Animals were clinically monitored for 10 days and swabbed weekly to establish Bsal loads for at least 14 days. Bsal DNA was demonstrated in skin swabs of all exposed animals, with increasing loads in three consecutive weekly samples (Experiment 6; Martel et al., 2014).

The transmission of Bsal from a presumed reservoir species to susceptible species was assessed by co-housing three *S. salamandra* 1:1 for 8 h with three infected *Cynops pyrrhogaster*, whose mean log₁₀ genomic load of Bsal was 1.68 ± 0.14 per swab. Animals were clinically monitored for 10 days and swabbed weekly to establish Bsal loads for at least 14 days. Bsal DNA was demonstrated in skin swabs of all exposed animals, with increasing loads in three consecutive weekly samples (Experiment 7; Martel et al., 2014).

3.3.1.3. Fulfilment of Koch's postulates

Although not invoked in Martel et al. (2013), in veterinary pathology, the assessment of causality of a pathogen for a particular disease often follows fulfilling Koch's Postulates. Table 2 presents Koch's

postulates (adapted from Koch, 1891) mapped against the procedures and evidence presented in Martel et al. (2013) (see sections above).

Table 2: Koch's postulates (adapted from Koch, 1891)

	Koch's postulate	Evidence in the case of Bsal causing chytridiomycosis in salamanders (presented in Martel et al., 2013)
1	The microorganism or other pathogen must be present in all cases of the disease	Confirmed by clinical examination of deceased wild animals from (1) the declining population taken into captivity (conservation programme), and (2) in experimentally infected animals following infection trials
2	The pathogen can be isolated from the diseased host and grown in pure culture	The pathogen was isolated and grown in culture from the deceased wild animals taken into captivity (conservation programme)
3	The pathogen from the pure culture must cause the disease when inoculated into a healthy, susceptible laboratory animal	The isolate was used in controlled infection experiments to cause disease of the same clinical pathology resulting in mortality as that observed in the source wild animals
4	The pathogen must be re-isolated from the new host and shown to be the same as the originally inoculated pathogen	The pathogen was re-isolated from 1/5 experimentally infected deceased specimens

The studies of Martel et al. (2013, 2014) thus present evidence to demonstrate Bsal as the causative agent of chytridiomycosis in salamanders as measured by the fulfilment of Koch's postulates.

3.3.1.4. Critical appraisal of the infection experiments

For the critical appraisal, information provided in the two publications and further clarifications on exposure and outcome details provided by the corresponding author were considered. In all seven experiments, the allocation of animals to study groups was randomised. In each experiment for each species used, control animals of the same species were used. No animals of the control groups died during the experiments. For all experiments, personnel were blinded and the experimental conditions were identical across all study groups for each of the seven experiments. For all seven experiments, an appropriate characterisation of the exposure and an appropriate assessment of the outcome were provided. No animals were withdrawn or excluded from any of the experiments. The main outcomes measured were reported for experiments 1–6, while for experiment 7 only the Bsal loads observed on the exposed animals, but not the results of the clinical monitoring were reported. In all experiments, biologically relevant effects were demonstrated. However, very small sample sizes were used in all seven experiments and most animals were derived from the same source populations. An overview of the critical appraisal results for the seven studies is given in Appendix D.

Based on the critical appraisal, the overall risk of bias in relation to internal validity of the studies is considered to be low. However, most of the animals tested in the seven experiments came from the same field or captive source populations, and are therefore expected to be correlated. In addition, the sample sizes used in the experiments were very low, ranging from one to eight animals per species. These numbers do not allow controlling for any potential confounders, and therefore, the results have to be considered as hypothesis-generating, rather than hypothesis-confirmatory.

Therefore, external validity should be assessed further in additional studies, to address the small sample sizes and the uncertainty regarding the representativeness of the animal subpopulations used in the experiments. It is recommended that the sample size of future experiments is designed, stating upfront the level of the effect considered as biologically relevant and its expected variability, the power that the experiment is intended to achieve and a significance level. In addition, it is suggested that the infection dose is fixed at a level that mimics infections under natural conditions.

Despite the statistical limitations related to the small sample sizes used in each of the individual experimental infection trials, resulting in low statistical power and failure, the critical appraisal determined to reject the Null Hypotheses on a case-by-case basis, with no difference in mortality rate between species. The critical appraisal further identified potential limitations relating to the 'representativeness' of the experiments, as most of the experimental animals originated from the same or a small number of source populations, and because it was unknown whether the experimental infection protocol (5,000 zoospores) represented an ecologically relevant exposure dose. However, despite small sample sizes: (i) statistically significant differences in mortality rates between exposed and control animals and between species cannot be ruled out in the absence of further analyses (e.g. by aggregating results

across multiple experiments using appropriate methods); (ii) several experiments involving the most sensitive species (e.g. fire salamanders) were biologically highly significant, consistently resulting in up to 100% mortality in the exposed vs 0% mortality in control treatments; (iii) the biological relevance of the outcomes of the infection experiments is considered to indicate that Bsal is likely to be biologically associated with disease (chytridiomycosis) and death of many infected salamanders, with infection outcomes following exposure (susceptibility to infection and disease severity) and mortality rates varying among species.

Based on the currently available evidence in the laboratory, it is likely that Bsal is a sufficient cause for the mortality of the susceptible species *S. salamandra* also in the field, and also of other species present in EU (see Appendix E).

3.3.1.5. Observational field studies

Swabs were taken in the field from 33 *S. salamandra* from the Dutch fire salamander population experiencing the decline during 2010, and from 51 fire salamanders not showing any clinical signs of disease from a population without a history of decline in Merelbeke, Belgium. The samples were examined by PCR for the presence of DNA of Bd and of Bsal. Bsal DNA was detected in 13/33 swabs from the declining Dutch population, but not (0/51) in the swabs from the Belgian population without a history of decline (Martel et al., 2013).

In addition, Bsal was detected retrospectively in remains of the epidermis of six wild fire salamanders that were found dead in 2010 or 2011 in the declining Dutch population and that were stored at -70°C (Martel et al., 2013).

3.3.1.6. Evidence from other studies

Blooi et al. (2015a) showed that high temperature treatment ($> 25^{\circ}\text{C}$ for at least 10 days) of wild ($N = 30$) and experimentally infected fire salamanders ($N = 25$) was able to eliminate Bsal infections, which was also confirmed by Sabino-Pinto et al. (2015). Blooi et al. (2015b) showed that wild ($N = 35$) and experimentally infected salamanders ($N = 30$) can be treated and cleared from Bsal with synergistic treatment with voriconazole, polymyxin E if being kept at 20°C during the treatment period (2 treatments per day for 10 days), eliminating the disease chytridiomycosis, as shown by real-time PCR.

Sabino-Pinto et al. (2015) further demonstrated that Bsal was associated with mortality of fire salamanders observed in a private captive collection of approximately 200 salamander individuals as determined via clinical and molecular diagnostics. They described mortality episodes in salamanders of multiple species (*S. salamandra*, *Salamandra algira*, *Salamandra corsica* and *Salamandra infraimmaculata*). Samples from 38 individuals from that collection were tested in three laboratories for the presence of Bsal and other potential pathogens (Chlamydiaceae, *Ranavirus*, Bd). Thirty-seven tested samples were positive for Bsal, but negative for the other pathogens.

In summary, Bsal has been demonstrated in clinical examinations of wild animals from declining populations, which died in captivity; it has been isolated and grown in culture from wild animals which died in captivity; it has been demonstrated to cause disease in experimentally infected animals with the same lesions found in dead wild animals, as shown by histopathology, and it has been re-isolated from experimentally infected, deceased animals. The fulfilment of all Koch's postulates indicates that Bsal is a primary agent of infection.

In addition, the evidence is supportive of the hypothesis that Bsal is a sufficient cause for the death of the susceptible species (*S. salamandra*).

Considering the presence of endangered species phylogenetically similar to the animals included in the experiments, it is likely that similar effects on the concerned species in the wild would occur, should they be exposed to Bsal.

3.3.2. Is the pathogen the only explanatory variable of the population decrease?

Ascribing causality of a population decline to a given pathogen with 100% certainty is difficult, particularly in wild situations without long-term and intensive field trials (e.g. mark-recapture and survival analysis) (e.g. Murray et al., 2009) or before-after data (i.e. being present in a site to monitor the invasion of the pathogen and observing the result directly on wild animals) (e.g. Lips et al., 2008). To date, in Europe, salamander population declines have preceded the detection of Bsal in wild animals, providing a natural obstacle in inferring categorically that declines were caused by Bsal and Bsal alone (Spitzen-van der Sluijs et al., 2016).

However, evidence has accumulated from multiple sources (including field observations combined with laboratory experiments, coupled with clear hypothesis testing procedures to eliminate potentially competing explanations), indicating that Bsal is a probable primary cause of specific population declines in at least some fire salamander populations in Europe, namely those in the Netherlands and Belgium (Spitzen-van der Sluijs et al., 2016), as described below.

3.3.2.1. Site specific evidence – declines of fire salamanders at Het Bunderbos, the Netherlands

In addition to the combination of lab and field evidence described in Martel et al. (2013), that identified Bsal in wild, declining amphibian populations, Spitzen-van der Sluijs et al. (2013) described the rapid population crash in the original wild amphibian population (~ 96% decline in population size over a few years) whose pattern is highly consistent with the invasion of a pathogenic organism. Similar population trajectories were a key feature in the early frog decline literature that implicated a pathogen as a potential causative agent, which was later discovered to be Bd (e.g. Laurance et al., 1996). The trajectories of population declines are known to differ in predictable ways depending on the cause(s) (Di Fonzo et al., 2013). Although the rapid decline curve is insufficient in itself to demonstrate a pathogen and specifically Bsal, as the responsible agent for the decline, the subsequent laboratory and experimental work by Martel et al. (2013) is consistent with the hypothesis that Bsal was a driver of decline in this case (see section above). However, most species that have had their conservation status assessed by the IUCN have multiple threats listed that are thought to contribute to their extinction risk (Maxwell et al., 2016). Hence, more studies on the role of Bsal as a primary or contributing factor in the context of other threatening factors (e.g., habitat loss, invasive species) in the observed population declines are needed (Murray et al., 2010).

Martel et al. (2014) further described lips evidence of severe disease outbreaks in Belgium in 2013 (Eupen, N 50°37'23"; E 6°05'19") and 2014 (Robertville, N 50°27'12"; E 6°06'11").

3.3.2.2. Multisite evidence

In addition to the evidence presented by Martel et al. (2014), multiple studies, albeit limited numbers of individuals having been used in these, suggest that multiple amphibian species and populations can be affected in different ways by Bsal. This variability is a complicating factor when considering whether Bsal is sufficient in itself to cause population declines in populations of European salamander species.

A similar pattern emerged with Bd, which, despite being highly pathogenic and capable of causing population declines, local population extirpation and extinction of susceptible species, is not pathogenic to all amphibian species and its pathogenicity is mediated by a very large range of ecological and life-history characteristics (Bielby et al., 2008).

Spitzen-van der Sluijs et al. (2016) described a survey covering 55 sites with free-living populations of newts and salamanders in Europe between 2010 and 2016. In total, 1,921 Caudata in three European countries were tested for the presence of Bsal. Forty-eight of the sites were chosen for sampling, based on reported amphibian deaths, negative amphibian population trends, or, after the new fungus had been detected and described, for preventive Bsal surveillance in susceptible populations or for geographic proximity to known Bsal outbreak sites. The sites were located in the Netherlands, Belgium, and in adjacent regions of the Eifel region in Germany (near the border with the Netherlands and Belgium). A further six sites in Germany and one in the Netherlands were also sampled in 2015, all of which were located > 100 km from the nearest known outbreak. Bsal was detected at 14 of the 55 sites, including both decline sites and sites in which declines had not been observed, and in animals of three different species (fire salamanders, alpine newts and smooth newts) (see Appendix E). There is thus coincidental/putative evidence from this survey that declines in European salamanders are consistent with the hypothesis that the presence of Bsal could at least partially contribute to population declines in populations other than the original sites described in the Netherlands (Martel et al., 2013; Spitzen-van der Sluijs et al., 2013) and Belgium (Martel et al., 2014). For details on the field sites where Bsal was detected, see Figure in Spitzen-van der Sluijs et al. (2016) and the technical appendix.¹⁶

As emphasised earlier, it is also important to note that Bsal is not the only cause of salamander population declines in Europe (see Appendix E). Furthermore, the presence of Bsal is not always associated with population declines. Therefore, the study above cannot ascribe causation between Bsal detection and declines, even where they occur together.

¹⁶ Available at <https://wwwnc.cdc.gov/EID/article/22/7/16-0109-Techapp1.pdf>

Spitzen-van der Sluijs et al. (2016) pointed out that 'the fungus may have been present at several sites before first detection' (e.g. where population monitoring before Bsal detection showed declines). This emphasises that a causal relationship between the presence of Bsal and the declines cannot be shown in these cases because no samples were collected before 2015. Furthermore, the presence of Bsal in populations is not always associated with population declines, despite the presence of dead and infected animals at those sites. Finally, clinical signs of mycosis were not always observed at Bsal-positive sites.

None of these observations, however, can rule out Bsal as a contributing factor to salamander declines in Europe. A lack of decline in the presence of the pathogen could have multiple explanations even if the pathogen is affecting the population (e.g. early stages of invasion with as yet undetectable impacts, or population resilience to the pathogen). Establishing whether Bsal is contributing to declines in these cases or not (i.e. ruling it out) would require much more intensive research, such as mark-recapture studies (Murray et al., 2009; Phillott et al., 2010). Variable impacts and slow declines due to Bd have been observed (e.g. Phillott et al., 2010) and Bsal could be equally devastating (i.e. resulting in population extirpation, extinction or contributing significantly to extinction risks) for some species even though this could be difficult to detect without robust studies.

A search for molecular evidence that the outbreak locations are connected could help establish whether Bsal has been introduced on multiple occasions or whether it is spreading from a single introduction site.

In summary: (i) in the context of multiple threats to salamanders in Europe, and in view of an absence of surveillance data on Bsal prior to its recent discovery, as well as a lack of detailed field studies to date, the strongest hypothesis at present to explain severe declines (99.9% reductions in abundance over 7 years) observed in at least one population of salamanders (fire salamanders at Het Bunderbos in the Netherlands) is the epidemic disease hypothesis (i.e. invasion by Bsal); (ii) Bsal is present in some declining populations. A direct or indirect contribution of Bsal to those declines cannot be ruled out and requires further investigation; (iii) Bsal has not been detected from some other declining populations. Other causes may be responsible for declines in these cases, but rigorous freedom of disease sampling would be required to rule out Bsal as a contributing factor in light of the associations noted above in other populations and in experimental infection trials; (iv) the presence of Bsal has been noted in populations regarded as not in decline in some cases. In these cases, Bsal may be (1) having no effect at the population level even where observations have been made of mortality due to infection in individuals; (2) having some effect at the population level that cannot be detected at present by current population monitoring methods. Contributions of Bsal to unobserved declines where Bsal is present cannot at present be ruled out (e.g. slow declines that have so far escaped detection due to lack of detailed field studies) and further detailed studies are required.

3.3.3. Amphibian species susceptible to Bsal

Experimental infections by zoospores of Bsal were used by Martel et al. (2014) to assess the host range of Bsal. Hundred sixty-one individuals of 35 amphibian species (2–8 individuals per species) were reported to be tested. The experimental tests showed variable responses to Bsal exposure among the tested amphibians. Based on the results, the authors sorted amphibians into four categories: resistant, tolerant, susceptible and lethal (see Table 3).

Table 3: Categorisation according to Martel et al. (2014)

Category	Description
Resistant (green)	Species that do not get infected by <i>Batrachochytrium salamandrivorans</i> (Martel et al., 2014), i.e. if the fungus invades the skin there is no detectable release of zoospores (as observed in the case of <i>Lissotriton helveticus</i> in the study, Martel (2016))
Tolerant (yellow)	Species that can get infected by the fungus, but never show clinical symptoms of disease, fungus releases zoospores to the environment
Susceptible (orange)	Species that get infected can show clinical disease and die of the disease, but some individuals are able to withstand the infection for prolonged periods of time and subsequently clear the infection
Lethal (red)	Susceptible species that get infected and all individuals die of disease

'Resistant' species were indicated in green; 'tolerant' species were indicated in yellow; 'susceptible' species were indicated in orange and 'lethal' species were indicated in red.

From the epidemiological point of view, the three categories 'tolerant', 'susceptible' and 'lethal' could be considered as potential spreaders, able to carry and disseminate the pathogen. 'Tolerant' and 'susceptible' species could carry infection without symptoms of disease.

None of the tested frogs and toads (Anura) were successfully infected; therefore, the whole order Anura has been suggested to be resistant to Bsal (Martel et al., 2014). The factors responsible for this are yet unsolved (Martel, 2016). The resistance of frogs to Bsal infection is further supported by several thousands of samples from wild, captive and museum specimens that were not found positive to Bsal (Martel et al., 2014; Zhu et al., 2014). For caecilians, the available data is very limited, as only one animal was tested which did not become positive (Martel et al., 2014).

In case of the tested salamanders, both the susceptible and lethal statuses regarding Bsal were observed; this variability shows a pattern consistent with biogeography and phylogeny of the group. The tested animals belonging to the family Salamandridae were mostly lethally susceptible to disease (European and American species); while animals from a lineage of susceptible species from Asia developed disease, but some individuals were able to eventually clear the infection. This Asian lineage is believed to contain species that were the original natural hosts to Bsal, before it was introduced to Europe (Martel et al., 2014).

Data on Bsal ability to infect salamanders was collected in wild populations, captive amphibians and museum collections (Martel et al., 2014; Sabino-Pinto et al., 2015; Spitzen-van der Sluijs et al., 2016). In several specimens from European and one African species, mortality was observed in infected animals (Sabino-Pinto et al., 2015; Spitzen-van der Sluijs et al., 2016; see also Appendix E), further supporting the experimental data. The data on other families is less clear. The American family Ambystomatidae (containing, e.g. the commonly traded and bred species *Ambystoma mexicanum*) might be resistant, as none of the tested animals of these species were successfully infected in experiments, nor found infected in nature or captivity (Martel et al., 2014; Zhu et al., 2014). Tested animals from species of the family Plethodontidae were either resistant or lethally susceptible to Bsal infection, but, given the size and geographic distribution of the family, great variability in response to Bsal can be expected (Martel et al., 2014). Tested individuals of two species of the Asian family Hynobiidae were resistant; while tested individuals of a third species of Hynobiidae were successfully infected in the experiment without developing symptoms of disease. Furthermore, members of this family were detected infected in wild populations in Japan (Martel et al., 2014). Some of the caudata families (Amphiumidae, Cryptobranchidae, Proteidae and Rhyacotritonidae) have not been covered sufficiently by the studies (Martel et al., 2014).

Appendix E provides an overview of the potentially susceptible salamander species, based on the results of animals from species that were either included in the experimental exposures to the pathogen or were found Bsal positive. The infection experiments involving salamanders have shown that individuals of the species *Paramesotriton deloustali*, *Cynops pyrrhogaster* and *Cynops cyanurus* are susceptible to Bsal, and individuals of the families Salamandridae, Plethodontidae, Hynobiidae and Sirenidae are tolerant: they can carry the pathogen without showing any clinical sign.

The only family from which none of the tested animals were found susceptible to infection, i.e. in which the infection with Bsal resulted in clinical disease with subsequent clinical recovery or death, is the Ambystomatidae (37 species), while all other salamander families have at least one species of which tested animals were shown to be either tolerant or susceptible (Martel et al., 2014; see also Appendix E). However, it should be noted that not all the species pertaining to this family have been studied so far.

These data should be interpreted with caution, as they are based on results from a small number of study animals, which mostly originated from the same source populations. In addition, the species that tested negative cannot be considered resistant.

3.4. Validity, reliability and robustness of the available diagnostic methods for the detection of Bsal (ToR 3)

3.4.1. Clinical signs of disease

Animals infected with Bsal may show changes in behaviour, e.g. anorexia, lethargy, apathy and ataxia (Martel et al., 2013).

The affected skin can show alterations from normal – changes in colouration, roughening, accumulation of sloughed skin and excessive sloughing. Martel et al. (2013) reported that, in the experimentally infected fire salamanders, the pathology consistently comprises multifocal superficial

erosions and deep ulcerations in the skin all over the body and cutaneous haemorrhages, as well as development of erosive vs hyperplastic/hyperkeratotic skin lesions. In fact, the name 'salamandrivorans' refers to the extensive skin destruction and rapid mortality observed in infected salamanders. All infected fire salamanders died within 7 days (Martel et al., 2013), but the mortality rate and the time at which the death occurs are likely to change across the different species (Martel et al., 2014).

Clinical signs of disease caused by fungi of the genus *Batrachochytrium* are in general variable and not pathognomonic, although the lesions linked to Bsal are characterised by marked skin ulcerations, as opposed to those caused by Bd, which typically induces epidermal hyperplasia and hyperkeratosis (Martel et al., 2013, 2014). As a consequence, clinical signs on their own do not appear to be a suitable means for diagnosis.

3.4.2. Microscopic diagnosis of Bsal presence

Microscopic observation of the fungus in the skin of infected salamanders is possible, but requires experience and proficiency. Possible sample types are skin scrapings or sections of skin from live salamanders, fresh dead specimens as well as formalin preserved specimens.

Histological examination is possible in dead animals, from natural mortality or after autopsy. Dead animals should be stored in 4–5% formalin. Classical histological examination is done using microscopic examination of paraffin-embedded, 5- μ m tissue sections, stained with haematoxylin-eosin, Ziehl–Neelsen or periodic acid shift.

In Martel et al. (2013), it is reported that keratinocytes with eosinophilic necrosis and marginated nuclei were at the periphery of the erosions. Each of these keratinocytes contained one centrally located thallus, the majority being segmented (colonial thalli). Transmission electron microscopic examination of the skin lesions confirmed the presence of intracellular structures consistent with the colonial thalli.

To improve the visibility of fungal cells, Congo red staining can be used. The intraepidermal organisms also stain with immunohistochemistry (Martel et al., 2013) as developed by Hyatt et al. (2007) to detect Bd. Immunohistochemical staining specific to Bsal is being developed and tested at Gent University (Blooï et al., 2016).

Although no specific study has been conducted with the aim of estimating sensitivity and specificity of the histological examination, these parameters are likely to be relatively low. In any case, considering the similarity of the lesions induced by Bd and Bsal, the basic histological examination as such cannot be used as a diagnostic test to discriminate between the two fungi.

3.4.3. DNA detection based methods – PCR

Several options of detection of Bsal using amplification of specific DNA sequences have been developed until now.

The internal transcribed spacer (ITS) 1 segment became almost universally used in DNA-based detection procedures of Bd (Annis et al., 2004; Boyle et al., 2004; Goka et al., 2009). The ITS 1 segment sequence variability actually leads to proposal of its use as marker gene for the 'The International Barcode of Life project' for all fungi (Seifert, 2009). The fact that the ITS 1 ribosomal DNA is present in each cell in multiple copies allows the genetic methods using it to theoretically reach sensitivity threshold under one cell per sample (Boyle et al., 2004).

3.4.3.1. Duplex real-time PCR

Martel et al. (2013) developed species-specific PCR primers that amplify the 5.8S ribosomal RNA gene and its flanking ITS regions: ITS 1 and ITS 2. The primers STerF and STerR amplifying 119 nucleotide long fragments developed by Martel et al. (2013) were shown to be specific to Bsal, not amplifying DNA from any of the tested Bd lineages. This assay provided very sensitive and specific (see below) evaluation of Bsal presence in the sample, but with no information on the abundance of the pathogen.

The pair of primers developed by Martel et al. in 2013 was further used in development of real-time quantification PCR assays and tested with SYBR green: the next step was designing and testing of specific fluorescent probe – internal oligonucleotide STerC (Blooï et al., 2013). The developed Bsal primers and probe were combined with the assay previously developed for detection of Bd by Boyle et al. (2004), thus providing duplex real-time PCR for the diagnosis of both pathogens in a single reaction (Blooï et al., 2013). The limit of detection (analytical sensitivity) of Bd/Bsal duplex real-time PCR is at the level of 0.1 genomic equivalent of a zoospore, thus allowing detection of the fungi in minute amounts (0.1 GE, retrievable in pre- or subclinical phases of the disease).

The original real-time PCR method of Bd detection developed by Boyle et al. (2004) was rigorously tested on specificity, sensitivity, repeatability and reproducibility (Hyatt et al., 2007) and was recognised as recommended detection method in the OIE Aquatic Manual¹⁷ after Bd was OIE listed. The duplex real-time PCR developed by Blooi et al. (2013) can be seen as an upgrade of the original Bd detection assay: a set of evaluations to optimise the diagnostic test were performed. In addition, the test has already been used in several laboratories providing consistent results (Sabino-Pinto et al., 2015). However, the samples used in the other laboratories were not the same, as required by the OIE guidelines (Manual of Diagnostic Tests for Aquatic Animals, Chapter 1.1.2 – principles and methods of validation of diagnostic assays for infectious diseases), compromising the fulfilment of criterion 2.3.1 (Reproducibility; see also Table F.2). In fact, the validation of the Bd/Bsal assay has completed the first 2 stages, but not the third stage as foreseen in the OIE guidelines. Based on the available data reported in Blooi et al. (2013), the test is, for the time being, eligible for a provisional assay recognition, which 'does not imply acceptance by the OIE' (OIE, 2016). More details can be found in Appendix F.

The main criticism in the validation process as described in the OIE Aquatic Manual is related to diagnostic sensitivity (DSe) and diagnostic specificity (DSp) of the test, which were not estimated or explicitly reported by the authors. However, based on the available data, it was possible to estimate those parameters. Martel et al. (2013) reported an experimental infection of five salamanders, all of which gave a positive test result; together with a set of five control individuals which gave a negative test result. The estimation of these two parameters was done using a classical approach (exact binomial test) and a Bayesian approach (assuming a uniform prior), and the results are reported in Table 4 and Figure 2. It can be seen that both approaches return a broad confidence interval which, in case of the exact binomial test, encompasses the value of 0.5. It has to be noted that these results are consistent with the reference table provided in the OIE guidance (table 2.1 of the Manual of Diagnostic Tests for Aquatic Animals manual) where it is indicated that the smaller sample size needed to estimate the actual DSe and DSp of a diagnostic test with a 90% level of confidence, assuming a prior value of 99% and allowing for an error equal to 5%, is 11. As a consequence, additional six samples could already improve the estimates enough to be statistically significant. Of course, the greater the sample size, the more precise will be the estimate.

Martel et al. (2013) also report the test results from fire salamanders sampled from a declining population (Het Bunderbos, the Netherlands (N50°54'51'', E5°44'59''), 2010). Thirteen salamanders out of 33 tested positive. Those data could also be used to estimate the DSe of the test by means of a latent class analysis, if the same sample had been tested with another diagnostic test.

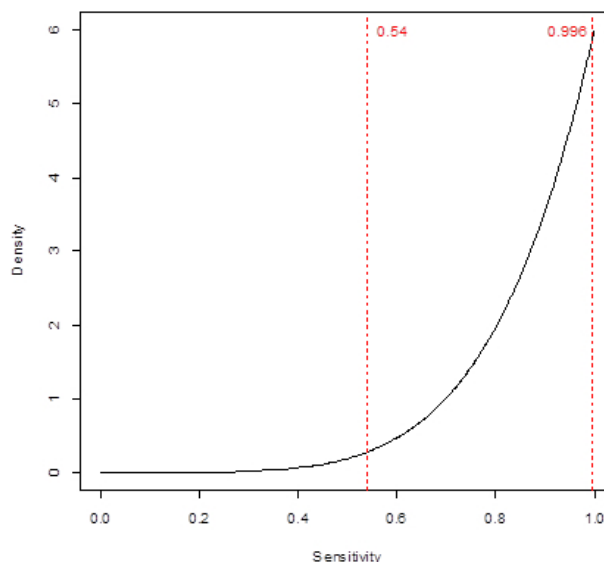


Figure 2: Beta distribution fitted to the available data from Martel et al. (2013). Sample size (truly infected animals) = 5, Positive results = 5. Prior distribution = $\beta(1,1)$

¹⁷ <http://www.oie.int/international-standard-setting/aquatic-manual/>

Table 4: Results of the estimation of the DSe and the DSp based on the available data as reported in Blooi et al. (2013)

	Sample	Bayesian approach ^(a)	Exact binomial
DSe	Positive samples = 5 Positive test results = 5	Median = 0.89 (95% CI = 0.54–1)	Prop = 1 (95% CI = 0.48–1); p-value = 0.06
DSp	Negative samples = 5 Negative test results = 5	Median = 0.89 (95% CI = 0.54–1)	Prop = 1 (95% CI = 0.48–1); p-value = 0.06

(a): Assuming a uniform prior.

Apart from the validation process and based on the estimates that current data lead to, it can be said, from an epidemiological point of view, that: (i) there are chances that the test either underestimates the actual prevalence (due to poor DSe) or overestimates the actual prevalence (due to poor DSp); (ii) the test could fail in detecting infected animals, as the probability that an infected individual is detected can reach very low values (~ 50%, i.e. 1 out of 2 in a worst-case scenario); (iii) the test could still fit for a freedom from disease framework, where DPs is assumed to be perfect (i.e. equal to 1) and the DSe can be taken into account, although a safe approach would imply a considerably high sample size (around 500, assuming a DSe = 0.5, a desired confidence of 95% and a population of 1,000 individuals). It has to be pointed out that these considerations are based on and due to the statistically limited sample size used in the validation process and do not necessarily reflect the actual performance of the test.

Additional information coming from the field has to be taken into consideration. The assay is being used by research teams and in veterinary practices. In case of the Bsal outbreak in a captive collection in Germany, three laboratories (Gent University, Institute of Zoology Zool. Soc London, and company Exomed) analysed the samples using the duplex real-time PCR assay and had consistent results in Bsal prevalence and infection intensity (Sabino-Pinto et al., 2015), suggesting good performance of the assay (reproducibility). The method has been already used also in several projects on Bsal detection in Europe, South America and United States (Bales et al., 2015; Bletz et al., 2015; Gimeno et al., 2015; Parrott et al., 2016; Balaz, 2017).

In conclusion, although the evidence produced so far does not afford a more precise estimate of the actual DSe and DSp, the data strongly suggest a good level of performance of the duplex real-time PCR.

3.4.3.2. Nested PCR

Nested PCR assay was developed by Zhu et al. (2014). The first step is based on the primer pair ITS 1f and ITS 4, which amplifies the 5.8S rRNA gene along with the flanking ITS of all fungi. The second step uses primers of Martel et al. (2013). This method is reported to have a lower detection limit – 0.01 equivalent of a zoospore, allowing Bsal detection even in, e.g. environmental samples. It has to be pointed out that the sensitivity of the test (i.e. the probability of detecting a positive case given an actual infection) has not been evaluated by the authors. In addition, 41 samples were taken from formalin fixed museum vouchers, which very likely contained degraded DNA, decreasing the probability of detection.

The samples that can be analysed by PCR methods include swabs, toeclips, skin sections, skin from moribund salamanders as well as environmental samples (water) (Blooi et al., 2013). The quantification of pathogen load allows distinguishing heavily infected individuals from those with low infection levels; this difference can indicate acutely diseased individuals or those with only subclinical infections. The real-time PCR assays are able to detect pathogen before the clinical signs of disease develop even in lethally susceptible species Martel et al. (2014). Clinical healthy, yet infected animals pose an important risk for introducing or spreading Bsal. At the moment, such individuals can only be identified with PCR assays.

3.4.4. Isolation and culture

Isolation of live culture is not a preferred method of Bsal diagnostics because the probability of successful cultivation of the fungus from infected salamanders is low (e.g. Martel et al. (2014) successfully re-isolated Bsal from one out of five experimentally infected *S. salamandra*). In case of *Batrachochytrium* fungi, cultivation is a complicated procedure requiring experience. The fungus is often overgrown by transient microorganisms present on amphibian skin (Longcore et al., 1999). The medium formula used for cultivation of both species contains gelatin hydrolysate, lactose and tryptone – sources of easily available sugars and amino acids.

The isolation/sampling and cultivation procedure has two main steps: (i) as the skin is in natural conditions inhabited by a complex set of various microorganisms (bacteria, protists, fungi), these need to be eliminated by cleaning in nutrient agar medium that contains broad spectrum antibiotics (penicillin, streptomycin) and (ii) the skin is then planted onto a new dish with a medium containing antibiotics. The second step follows as soon as the growth of sporangia or the presence of mobile zoospores is detected. The sporangia are replanted onto a new medium, free of antibiotics. The replanting can be done either on a new agar medium or into a broth (Longcore et al., 1999; Martel et al., 2013).

Considering the complexity of this technique, isolation of live culture is not the most suitable method for the diagnosis of Bsal, although it represents a step allowing further typing of the pathogen and is an important step in research.

3.5. Effectiveness and feasibility of a movement ban (including intra-EU trade and introduction from non-EU countries) of traded salamanders, including both Asian and non-Asian species (ToR 2)

3.5.1. Description of the trade

The amphibian trade takes place for various purposes, including trade of animals for use as pets, consumption (mostly frog legs), medical use (traditional medicine) and research. These movements involve hundreds of species, including many Caudata, almost worldwide.

The commercial trade of the vast majority of the world's amphibian species is not regulated and they can therefore be freely shipped. In fact, only 3.4% of all amphibian species are currently listed in the CITES Appendices and/or EU wildlife Trade Regulations-Annexes (Auliya et al., 2016).

The main purpose of the trade of salamanders is commerce, as confirmed by the data reported in the CITES Trade Database (see following paragraphs and Appendix G).

Southeast Asian newts have been found for sale online as pets in several European countries: Austria, Germany, Italy, the Netherlands, Poland, Spain and the UK (Rowley et al., 2016). In the UK, the mean number of pet-shop licenses issued by local councils, and specifically of those that permit the sale of amphibians, showed an increasing trend in the years 2000, 2005 and 2010, which would suggest growth in the pet trade sector over that time (Wombwell, 2014).

The complexity of the amphibian pet trade was described by Wombwell (2014). It involves a wide range of both captive-bred and wild-caught species, originating from multiple countries and involving an estimate of six million amphibians per year (OIE, 2006).

Salamanders are traded at all life stages. The mode of transport is highly variable, and depends on various criteria, e.g. country of export, order of request, business relationships, level of enforcement, trade routes, IATA guidelines,¹⁸ etc. (Auliya, 2017).

For an epidemiological assessment, it is considered important to distinguish between direct and indirect imports of salamanders: (i) a 'direct import' refers to individuals imported directly from its country of origin (e.g. from Japan to Germany); whereas, (ii) 'indirect imports' concern individuals that are imported indirectly via another country (e.g. from China to Spain via Hong Kong-SAR¹⁹). Trade routes may also depend on species and countries of origin/export. The epidemiological relevance of this difference lies in the possibility of salamander species to be cross-contaminated while sharing water or facilities in the 'stopover' sites.

In the CITES Trade database, the main providers to EU-28 of caudata species listed in CITES Appendices and/or Annexes of the EU Wildlife Trade Regulations between 2005 and 2015, are estimated to be China, United States, Hong Kong-SAR and Japan (see Appendix G). From the same data, individuals that were imported into EU-28 mainly for 'commercial purpose' (59.45% of the direct import and 95.62% of the indirect import) were traded primarily from 'unknown sources'. The main 'known sources' reported are estimated to be 'Animals bred in captivity' for both direct and indirect imports (28.77% of the direct import and 22.01% of the indirect import), whereas 'Specimens taken from the wild' are estimated to be mainly subjected to indirect trade (23.67%). A low percentage of specimens taken from the wild was reported for the direct trade (2.98%). For more detailed data, see Appendix G – Table G.1).

¹⁸ <https://www.fws.gov/policy/library/2016/2016-00452.pdf>

¹⁹ Hong Kong Special Administrative Region of the People's Republic of China.

3.5.1.1. Extent of the trade of Caudata

The data on the number of Caudata imported into the EU collected from Members States through the EFSA AHAW Network (see Section 2.1.2) were fragmented and without sufficient granularity for the analyses required for this report.

The lack of trade data is reported by several authors (Yap et al., 2015; Rowley et al., 2016) and it was highlighted that the lack of a unique identifier (code) for amphibians makes it difficult to trace flow of the European amphibian trade (Auliya et al., 2016).

Data on salamander imports in the Netherlands were reported by Spitzen-van der Sluijs et al. (2015; see Table 5), providing an indication of the actual size of the salamander imports per year at MS' level.

Table 5: Data on salamanders imported into EU derived from Spitzen-van der Sluijs et al. (2015)

Species, number and time period	Origin	Destination	References
21,000 individuals, <i>Paramesotriton chinensis</i> , <i>Notophthalmus viridescens</i> , <i>Cynops</i> sp. (2013)	Asia, North America (<i>Notophthalmus viridescens</i>)	the Netherlands	Spitzen-van der Sluijs et al. (2015)

Based on the available trade data of Caudata in the CITES Trade database, for the period of 2005–2015, it was estimated that 61 importing movements occurred into EU-28 for a total number of 4,867 individuals (including live, specimens, skeletons and bodies) as reported by the importers; of these estimates, 22 importing movements and 1,119 individuals regarded species listed in CITES Appendices (see Appendix G).

However, the CITES Trade database includes only the caudata species that are listed in the CITES Appendices and/or EU wildlife Trade Regulations-Annexes, i.e. 37 species pertaining to five families (see Appendix A), which represent approximately 5% of the total number of species of Caudata (703 species pertaining to nine families (see Appendix E)). Therefore, for 95% of the world's caudata species the commercial trade is not regulated and related data are limited, patchy and not harmonised.

In the case of import into EU-28, trade was reported by importing countries for only 10 species of Caudata (pertaining to Ambystomatidae, Cryptobranchidae, and Salamandridae; see Appendix G); Wombwell (2014) emphasised that proportionally very few of the amphibian species in trade are CITES-listed species. Therefore, these data are just a part of the overall estimate of the trade of Caudata into EU.

The US routinely record amphibian imports and exports on the LEMIS (i.e.: the USFWS (US Fish and Wildlife Service)) LEMIS data (Law Enforcement Management Information System) (Wombwell, 2014).

Approximately 156,000 salamanders are reported to be annually imported into the US, mostly including shipments with a Bsal risk (originating from Asia or crossing Asian ports; Yap et al., 2015). In 2014, the market value of salamanders imported to the US was estimated at US\$ 924,707 and salamander species represented 5.5% of the amphibians imported into the US from 2004 to 2014 (Gray et al., 2015).

The import into the US of Caudata regards the species listed in CITES Appendices pertaining to the families Ambystomatidae, Cryptobranchidae and Hynobiidae. From the CITES Trade database, the estimated number of imports into the US of caudata species listed in CITES Appendices was 31 importing movements for a total of 1,536 imported individuals (including eggs, specimens, live, skeletons, bodies and meat as reported by the importers). None of the Salamandridae listed under CITES Appendices were reported to be traded into the US between 2005 and 2015 (see Appendix G).

The numbers of live salamanders imported to the US was studied for the period of 2010–2014. The total gross imports were 779,002 salamanders. About 99% originated from Asia, and 98% were species native to Asia (Yap et al., 2015; data source: USFWS).

Comparing the information reported in the CITES Trade database regarding the individuals imported into the US between 2010 and 2014 and the data of Yap et al. (2015), it appears that CITES official data represent approximately 0.18% of the total value. Assuming US government data on salamander trade is robust, these figures were used to extrapolate an estimate on the real imports into EU-28. Based on this assumption, between 2005 and 2015, it is estimated that a total number of 620,000 individuals have been imported into EU-28 (see Appendix G).

In addition, illegal movement appears to be present. Reported illegal activities include collection within nature reserves and laundering of wild-caught animals as captive-bred (Auliya et al., 2016; and

references therein). Online searches revealed reportedly wild-caught Southeast Asian newts for sale belonging to species that were not recorded as having been imported into the EU; this would indicate an unknown proportion of illegal trade into the EU (Rowley et al., 2016). Hence, the figures given in the previous paragraphs are probably minimum ones, and the real number of imports of salamanders into the EU is likely to be higher.

3.5.2. Epidemiological and technical considerations on movement bans

3.5.2.1. Justification for movement bans

Due to the risk of pathogen introduction posed by amphibian translocations including international trade, several authors have pointed at trade-bans, specifically of Asian Caudata, as a suitable tool for risk management (Gray et al., 2015; Yap et al., 2015; Rowley et al., 2016). Commercial trade in salamanders was in fact considered to be the most likely pathway for entry of Bsal into new geographical regions (Yap et al., 2015; Grant et al., 2016a).

Movement bans constitute key risk mitigation measures to prevent (human-driven) pathogen spread into naïve areas and populations, particularly as management of invasive pathogens becomes difficult once they are established in wildlife populations. Therefore, import restrictions to limit pathogen introduction, and early detection through surveillance of high-risk areas are priorities to control pathogen invasion (Gray et al., 2015; Richgels et al., 2016). In parallel, by stemming the trade of Asian newts to Europe and North America, the risk of extirpations of salamander populations can be decreased (Stuart et al., 2014).

In the US, Bsal has not been detected (Muletz et al., 2014; Bales et al., 2015). A trade ban has been set up under the Lacey Act (18 U.S.C. 42),²⁰ listing 201 salamander species, due to risk of Bsal. The listing became effective on January 2016 and the species that have been listed pertain to 'the 20 genera where at least one species has been positively identified as a carrier of Bsal and there is no countervailing conclusive evidence suggesting that some species within the genus are not carriers'. The Canadian government is actively working to reduce the risk of Bsal introduction through import control, by exploring emergency measures similar to those being considered in the US (Gray et al., 2015). A ban on the importation of all salamander species into Switzerland has also been established by the Swiss Federal Food Safety and Veterinary Office (Gray et al., 2015).

Although Bsal has already been introduced in the EU (detection of Bsal in EU wild populations, in imported salamanders and in salamanders kept in captivity; see also Section 3.1), a ban of intra-EU movements of salamanders could limit the spread of the fungus to new areas and MSs.

Restrictions of Asian caudata trade to EU have been suggested to protect native European salamanders from further introductions/outbreaks of Bsal (Rowley et al., 2016; UNEP-WCMC, 2016). Regarding the trade from Asia as a viable means for Bsal introduction and spread, it should also be taken into account that a survey on a limited sample of individuals from China (49 salamanders from food markets or farms and 41 from formalin preserved specimens, i.e. museum vouchers), conducted by Zhu et al. (2014) did not detect Bsal in any of the specimens. However, the sensitivity of the test used was not reported, and it should be considered that the DNA originating from museum vouchers is usually very poor, compromising the validity of the results (see also Section 3.4). A visual inspection of 366 salamanders (*Paramesotriton chinensis*, *Notophthalmus viridescens*, *Cynops* sp.) imported into the Netherlands and tested in trade did not show evidence of Bsal infection (Spitzen-van der Sluijs et al., 2015). However, the findings of these two papers do not exclude trade from having a role in the introduction and further spread of the fungus.

Three Asian salamander species have been suggested to serve as reservoirs for Bsal: *Cynops pyrrhogaster*, *Cynops cyanurus* and *Paramesotriton deloustali* (Martel et al., 2014). However, additional species have shown the potential to carry Bsal (see Section 3.3.3).

Should a movement ban be considered, as a safer option, all the families gathering at least one species with individuals that have shown to be tolerant (independently from the level of pathogenicity, see Table 3) could be included, i.e. Salamandridae, Plethodontidae, Hynobiidae and Sirenidae. These families contain 647 species out of the 703 caudata species (see Appendix E).

²⁰ US have prohibited 'the importation, transportation, or acquisition of any live or dead specimen, including parts, but not eggs or gametes, of the genera *Chiglossa*, *Cynops*, *Euproctus*, *Hydromantes*, *Hynobius*, *Ichthyosaura*, *Lissotriton*, *Neurergus*, *Notophthalmus*, *Onychodactylus*, *Paramesotriton*, *Platodon*, *Pleurodeles*, *Salamandra*, *Siren*, *Taricha*, *Triturus* and *Tylotriton*'. For full text: Federal Register/Vol. 81 No. 8, 13 January 2016 – Rules and Regulations, Fish and Wildlife Service (50 CFR Part 16); available at: <https://www.fws.gov/policy/library/2016/2016-00452.pdf>

The fact that salamanders can originate either from wildlife or from captivity (farms, hobby breeders, pet traders, etc.) is an aspect that should be considered when assessing the feasibility of a trade ban.

Wild Hynobiidae are distributed in Asia; the species pertaining to the family Sirenidae and some species of the families Salamandridae and Plethodontidae, in the wild, have their distribution in North America (see Table 1). In addition, species of these four families are bred in captivity also in Asia. Independently from the geographical origin, there is a risk for cross-contamination with Bsal, when individuals of these families are traded from kept communities (or from unknown sources) and/or through indirect trade. The probability of such a scenario is likely to be small, but not null.

Therefore, there is the possibility of Bsal being transmitted between salamander species native to different areas during transportation, in the breeding facilities or in the hobbyist collections. This makes the risk of Bsal occurrence in traded amphibians somehow independent from the situation in the wild and it complicates the picture.

Nonetheless, it should be noted that there is no precise distinction in general between the sources 'wild' or 'captive-bred', which largely depends on the species, and some species reported as captive-bred may be laundered as such (Auliya, 2017).

As already discussed, the family Ambystomatidae, (37 species), can be considered resistant, although not all the species pertaining to this family have been studied so far. In addition, even if originally from North America, Ambystomatidae are bred in captivity also in Asia, where they could get cross-contaminated, and spread Bsal via trade. Therefore, the risk of traded Ambystomatidae to carry Bsal is not null, but based on the present state of knowledge, the species of the family can be considered an unlikely host of Bsal and possibly excluded from the list of the banned species.

3.5.2.2. Factors affecting effectiveness and feasibility of a movement²¹ ban

For the UK, Wombwell (2014) listed trade bans under the unfeasible options because (i) the current data is considered not enough to justify a ban on imports based on potential disease dissemination; (ii) the UK is not yet equipped to enforce a ban at all points of entry. Additionally, it would be problematical to ban trade for some, but not all uses, and it would potentially increase the illegal trade.

Auliya et al. (2016) envisaged a (temporary) trade ban that includes the species fulfilling at least one of the following criteria:

- Species listed as critically endangered, endangered or vulnerable in the IUCN Red List of Threatened Species.
- Species with an extent of occurrence²² < 20,000 km² or area of occupancy²² < 20,000 km².
- Species that are nationally protected in their country of origin.

In order to include relevant information related to the effectiveness and the feasibility of a movement ban, the EFSA working group (WG) of experts were asked to list the major factors that could influence the implementation of such a measure. A qualitative estimation of the degree of uncertainty was assigned to each of the factors, together with a qualitative evaluation of the impact on the feasibility and the effectiveness of a movement ban.

The first outcome of this evaluation (see Table 6), is the high degree of uncertainty related to almost all the factors having a role in the implementation of a movement ban. This lack of knowledge, at this point in time, makes it impossible to make strong conclusions on the parameters of interest (i.e. effectiveness and feasibility of a movement ban). For example, the real trade volume is actually not known, as already described above. Therefore, possible scenarios with the related degree of feasibility and effectiveness as perceived by the WG experts have been outlined. Table 6 summarises the main relevant factors and provides arguments on the feasibility and effectiveness of a movement ban (including intra-EU trade and introduction from non-EU countries) of traded salamanders, including both Asian and non-Asian species for each possible scenario.

²¹ The term 'movement' refers to both movements of individuals within countries and between EU MSs. The term 'trade' refers to movements of individuals from/to third countries. In order to assess the effectiveness and feasibility of movement bans, also data and evidence regarding trade bans has been used. Where such references used the term 'trade', it was also used in this report.

²² IUCN Red List Categories and Criteria. Version 3.1 (second edition). Available at <http://www.iucnredlist.org/technical-documents/categories-and-criteria/2001-categories-criteria>

In summary, it can be said that if trade volumes are actually as high as the estimates suggest (see Section 3.5.1.1), a movement ban may be difficult to implement due to the high demand on resources that controlling large shipment volumes causes. However, the potential to reduce large numbers of animals moved through bans is also high. On the other hand, if trade volumes are much lower than estimated, a ban could be considered easier to implement. However, the volume reductions of a movement ban would be more limited.

A species-specific movement ban would require detailed knowledge on which species are susceptible to the pathogen. However, such knowledge is currently limited as susceptibility testing has not yet taken place for all species, and low sample sizes in tested species did not allow achieving statistically significant results (see Section 3.3). A species-specific movement ban would require accurate identification (via taxonomic training) among enforcement personnel to be effective. Should a movement ban be considered, due to the complexity of the taxonomy as well as the lack of current evidence related to which species are susceptible, a movement ban at the level of taxonomic order is likely to be both more effective and more feasible.

The possibility that Bsal remains viable outside susceptible/tolerant species (e.g. on fomites, travel boxes, etc.) is estimated to be low, but cannot be ruled out at present. Therefore, an effective movement ban also needs to consider the possibility of cross-contamination taking place during transportation, in breeding facilities or in hobbyist collections, as the risk of introduction of Bsal is not only determined by the geographic origin of the traded susceptible or tolerant species, but also by the Bsal infection status within trade routes.

In summary, it was assessed that the feasibility of a movement ban mainly depends on the import volumes; the effectiveness of a movement ban is mainly dependent on: (i) import volumes, (ii) possibility of Bsal to remain viable outside susceptible/tolerant species (e.g. fomites, travel boxes, etc.), and (iii) capacity to limit illegal movements.

Table 6: List of factors, with related uncertainty, affecting feasibility and effectiveness of a movement ban

FACTORS affecting the effectiveness and feasibility of a movement ban	UNCERTAINTY on the knowledge	IMPACT on FEASIBILITY of a movement ban on introduction and spread	IMPACT on EFFECTIVENESS of a movement ban on introduction and spread
Import volumes	High uncertainty – volume is probably high, but very little data is available (Section 3.5.1.1)	If volumes are actually high, a movement ban will be difficult to implement as controlling large volumes of shipments requires a lot of resources Should the volumes be low, the ban could be considered feasible	If trade volumes are actually as high as the estimates suggest, a movement ban may be difficult to implement. However, the potential to reduce large numbers of animals traded through bans is also high. On the other hand, if trade volumes are much lower than estimated, a ban could be considered more feasible. However, the volume reductions of a trade ban would be more limited
Possibility of Bsal to remain viable outside susceptible/tolerant species (e.g. fomites, travel boxes, etc.)	High uncertainty – the possibility is estimated to be low, although not null. Very limited or no evidence available (Section 3.5.2)	No impact on the feasibility	If the probability that Bsal remains viable outside the banned species (e.g. fomites, travel boxes, etc.), the effectiveness of a movement ban would be jeopardised The possibility of cross-contamination taking place during transportation, due to the Bsal infection status within trade routes, has to be considered
Knowledge of the relevant species that need to be covered by the movement ban	High uncertainty – not all species have been studied yet. The susceptibility related to the species that were not included in any study is the outcome of extrapolation from experiments (Section 3.3.3)	The impact on the feasibility is high if the movement ban has to be species-specific, but low if the movement ban addresses the level of the taxonomical order	A species-specific movement ban would further require accurate identification (via taxonomic training) among enforcement personnel to be effective The effectiveness would be low in case the movement ban is species-specific, but very high if it addresses the level of the taxonomical order
Illegal movement of susceptible animals	High uncertainty – appears to be present, but little is known (Section 3.5.1.1)	No impact on the feasibility of a movement ban as this would pertain only to the legal movements	The impact on the effectiveness of a movement ban is actually incalculable as no data will ever be available. The presence of an illegal movements itself would be sufficient to affect the effectiveness of any official ban

3.6. Possible alternative methods and feasible risk mitigation measures to ensure safe international and EU trade of salamanders and their products (ToR 4)

3.6.1. Introduction on alternative risk mitigation measures

Bsal has been reported in some but not all MSs (see Section 3.1). Therefore, in some areas of the EU, the focus might be to monitor the presence/absence of the fungus and the prevention of entry, whereas in other areas the focus might be to control the fungus to prevent further spread.

A rapid decline in fire salamanders (*S. salamandra*) has been reported (Spitzen-van der Sluijs et al., 2013). Further analysis is required on the role of Bsal and other biological, chemical and physical stressors in population decline and possible extinction of salamander species in Europe (see Appendix H). In a meta-analysis of amphibian population trends in North America, it was found that amphibian individuals are being lost from local populations at an average rate of 3.79% per year. However, these declines were not related to any particular threat at the continental scale. Hence, the authors stressed that a greater emphasis on local solutions to this globally shared phenomenon was needed (Grant et al., 2016b; but see also Semlitsch et al., 2017). Options for wildlife disease control have been reviewed (Gortázar et al., 2014). Disease control can be achieved by different means, including preventive actions, host population control, habitat management and/or treatment. However, not all options are suitable for all host-pathogen pairs. The alternative options of zoning or no-action²³ should also be considered, particularly in view of a cost/benefit assessment. Ideally, several tools (e.g. medication + population management) should be combined in an integrated control strategy. More information can be found, for instance, in reviews of Gortázar et al. (2014) and Langwig et al. (2015).

Both reviews stress that wildlife disease management should be adaptive and the efficacy of different strategies should be assessed concurrently with implementation to inform future management policies and to re-evaluate initial decisions on intervention.

Risk mitigation options in amphibian infectious diseases have been reviewed (e.g. Scheele et al., 2014; Grant et al., 2016b). These include:

- 1) trade-bans (see Section 3.5) and related means (e.g. certification of disease-freedom for salamander importation) to mitigate risks due to amphibian translocations;
- 2) other preventive actions, such as quarantines and hygiene guidelines for working on-site with wild amphibians;
- 3) immediate disease management actions;
- 4) habitat manipulation;
- 5) treatments, including *in-situ* medication;
- 6) other options, such as selective breeding for tolerance or resistance.

3.6.2. Alternative risk mitigation measures to ensure safe international and EU trade of salamanders

In 2015, a formal working group led by Amphibian Research and Monitoring Initiative (ARMI) scientists from the US Geological Survey (USGS) Patuxent Wildlife Research Center, Fort Collins Science Center, and Forest and Rangeland Ecosystem Science Center, was held at the USGS Powell Center for Analysis and Synthesis in Fort Collins, Colorado (Grant et al., 2016b). The US WG produced a list of potential action categories considered for Bsal management (in order to get prepared in case of Bsal detection) (see grey cells of Table 7). These and additional measures proposed by the EFSA WG experts were assessed for their relevance (Table 7).

The measures identified as relevant, other than a movement ban (see Section 3.5), are briefly described below. More detailed analyses would be required if risk managers would consider their implementation. Some background information is provided in Appendix I – on measures that were considered not relevant to ensure safe trade of salamanders.

Mitigation measures considered relevant for ensuring safe international and EU trade of salamanders.

²³ If the 'no-action' option is chosen, disease and host monitoring should still take place, as in the future monitoring might indicate a more critical situation (regarding the population status or the disease situation), which might change the decision whether to intervene, or keep on with 'no-action'.

Table 7: List of potential measures considered for *Batrachochytrium salamandrivorans* (Bsal) management that have been assessed by the EFSA WG experts for their relevance in ensuring safe trade of salamanders – based on Grant et al. (2016a)

Mitigation measures reported in Grant et al. (2016a) ^(a)	Relevance to ensure safe international and intra-EU trade (assessed by the EFSA WG experts)
Containment of infected sites	No
Alter host species composition	No
Apply anti-fungal agents to salamanders	No (theoretically relevant, but not economically viable)
Deploy Bsal zoospore removal methods	No
Remove susceptible and tolerant salamanders from infected sites	No
Limit site access (by humans and other vertebrates)	No
Quarantine salamanders	Yes
Require health certification	To be further investigated
Apply anti-fungal agents to habitats	No
Vaccinate salamanders	Not in the absence of a vaccine
Apply probiotics to salamanders	No
Physical modification of habitat	No
Enforce fieldwork biosecurity	No
Create assurance colonies ^(b)	No
Breed salamanders for resistance and/or tolerance	No
Enact legislation that authorizes actions on wildlife pathogens	Yes
Ban all importation of salamanders	Yes (see Section 3.5)
Restrict salamander trade	Yes
Destroy habitats of infected sites	No
Additional measures identified by the EFSA WG experts	
Tracking all traded species	Yes
Hygienic procedures	Yes
Increase public awareness	Yes

(a): Grey cells indicate the list of potential action categories considered by the US WG for Bsal management (in order to get prepared in case of Bsal detection).

(b): Assurance colony: a captive population of a critically endangered species that is being carefully managed and bred for long-term survival of the species.

Applying antifungal agents to salamanders before movement of animals can clear infections with Bsal and is considered to be the only way to remove infectious Bsal particles from an animal. A treatment protocol has been described by Blooi et al. (2015b). However, this measure might not be commercially viable due to the cost this would incur.

Quarantining salamanders is considered a relevant measure for safe trade. Sainsbury et al. (2016) successfully used quarantines to manage disease risks during pool frog releases in the UK. Post-release health surveillance was carried out through regular health examinations of amphibians in the field at the reintroduction site via collection and examination of dead amphibians. This measure could be applied for both imports into the EU and intra-EU movements. The recommended duration of quarantine is 6–8 weeks during which the salamanders are to be sampled by skin swabs and tested by PCR assay at the beginning and the end of quarantine, although the test, at this point in time, has not been yet validated and the uncertainty related to its performance is very high.

For illustrative purposes, Table 8 reports some examples related to the sample size needed in order to estimate, with a 95% confidence, that the proportion of animals infected by Bsal is below 1% (i.e. free from Bsal²⁴), based on the number of animals included in the consignment and assuming a DSe

²⁴ For mathematical reasons, the threshold (design prevalence) cannot be set at 'zero'. A prevalence value of 1% is arbitrary, but usually well accepted for other diseases (e.g. *E. multilocularis* infection in dogs and related EU legislation).

equal to 0.5 (i.e. close to the worst-case scenario). The approach is well documented in the scientific literature and known as 'output based surveillance' (Cameron, 2012). It has to be noted that the consignment cannot include less than 432 animals: a smaller consignment will lead to a confidence below 95%. In practice, if a consignment includes 500 animals, 451 of these animals should undergo the duplex real-time PCR. Should all of them test negative, the consignment can be considered 'free' from Bsal (i.e. the number of animals infected by Bsal in the consignment is, with a 95% confidence, equal or below 4). More scenarios and different parameters can be set according to the needs. For more options, it is possible to refer to the open access tool developed by Varewyck et al. (2016).

All salamanders are to be checked for clinical signs of disease and necropsy of dead individuals should be mandatory (Mutschmann, 2015).

Table 8: Sample size needed to estimate with a 95% confidence that the proportion of animals infected by Bsal is below 1%, based on the number of animals included in the consignment and assuming $DSe = 0.5$

Consignment size	432	500	700	1,000
Sample size	432	451	487	517

Requiring animal health certificates to ensure safe trade has been considered a relevant measure for other animal species (e.g. honey bees have to be free from small hive beetle when they are imported into the EU from third countries); however, in the context of Bsal and salamanders, this alternative mitigation measure needs to be further investigated.

Enacting legislation that authorises actions on Bsal, specifically requiring that animals be tested in order to demonstrate freedom from Bsal before movement can take place, could also ensure safety of trade.

Restricting salamander movements is an alternative to a movement ban. Restrictions on salamander movements could be achieved by listing identified host species (see Section 3.5). Thirty-seven caudata species are currently listed in the CITES Appendices and/or in Annexes of EU Wildlife Trade Regulations and they pertain to five families (for the full list, see Appendix A). Listing more species has been identified as relevant measures by several authors (see for example, Yap et al., 2015; Auliya et al., 2016; Rowley et al., 2016; UNEP-WCMC, 2016). It is important to note that listing the species in the Annexes B, C and D of the EU Wildlife Trade Regulations does not mean an immediate ban of trade in these species, but only introduces the obligation to present certain documents when trading in these species. In practice, listing of species discourages persons to move these species due to the labour-intensive administrative procedure. On the other hand, it might also increase illegal movements of banned species (Grant et al., 2016a). Overall, this measure might help to reduce movements of susceptible salamanders, hence the risk of Bsal introduction, but is recommended to be applied in combination with other measures described in this section.

Tracking all traded species by, e.g. setting up a mandatory international system that will track all traded species (Yap et al., 2015), would help in identifying the sources of Bsal and thereby provide valuable indication as to in how far trade rules need to be modified. Concerns remain about the practical implementation.

Hygienic procedures as concerns biosecurity measures before and during movements are considered to provide for safe trade. Within-site hygiene protocols are published, aiming to reduce the risk of transmission among individuals and spread between sites/populations (e.g. Murray et al., 2011). These measures include aspects such as handling and holding of amphibians, skin disinfection before and after invasive procedures, and treatment of equipment (Phillott et al., 2010). These measures might also help in achieving safe trade but it is recommended that they are applied in combination with other measures described in this section.

Increasing public awareness by informing the public and specifically pet-owners and traders, in order to involve them in disease detection and disease control (Langwig et al., 2015) achieves better compliance with safe trade rules. This measure might also increase the early Bsal detection capacity. Combining this measure with other measures is recommended.

The measures listed above, have been considered relevant to safe international trade (imports into the EU) and intra-EU movements. However, further analysis of these measures is required before

recommendations can be made regarding their implementation for imports into the EU and for intra-EU movements.

Animal by-products that are obtained from salamanders which underwent heat treatments at 25°C for at least 10 days (Bloom et al., 2015a) are not considered relevant for the spread of Bsal to the salamander populations in the EU, as Bsal is not able to survive in such temperatures. Further, the fungus is dependent on water and desiccation is fatal to all life stages (Johnson et al., 2003; Van Rooij et al., 2015). Therefore, desiccated salamander by-products can be considered to not play a role in the spread of Bsal to the salamander populations in the EU.

Conclusions

ToR 1: Assessment of the potential of Bsal to affect the health of wild and kept salamanders in the Union

- The available scientific evidences fulfil the Koch's postulates, indicating Bsal as a primary agent of infection.
- The evidence for a causal relationship between the death of salamanders due to infection with Bsal and population declines in the wild remains limited.
- Despite the statistical limitations related to the small sample size of the experimental infections of salamanders with Bsal, leading to a low statistical power and the impossibility of rejecting the Null Hypothesis for each of the individual studies and per species comparison, the biological relevance of the outcomes is considered to indicate that Bsal is biologically associated with death of salamanders.
- Based on the currently available evidence, it is likely that Bsal is a sufficient cause for the death of at least one susceptible species, *S. salamandra*, both in the laboratory and in the wild.
- Despite small sample sizes, the experimental evidence to date further indicates that Bsal is associated with disease and death in 12 European and in 3 Asian salamander species, and is associated with high mortality rate outbreaks in kept salamanders. Experimental infection by Bsal was successful in at least one species from each of the families Salamandridae, Plethodontidae, Hynobiidae and Sirenidae.
- Bsal is present in some declining populations, but was not detected in some other declining populations. In addition, Bsal has been noted in populations regarded as not in decline. Better monitoring of both pathogen and population is needed for a better understanding of the association.

ToR 2: Effectiveness and feasibility of a movement (including intra-EU trade and introduction from non-EU countries) ban of traded salamanders, including both Asian and non-Asian species

- It appears that official data represent approximately 0.18% of the total trade value. Based on this assumption, between 2005 and 2015, a total number of individuals imported into EU-28 can be estimated to be around 620,000. Other data currently available are fragmented and without sufficient granularity for analysis.
- Illegal movements of salamanders are likely to also occur; however, the magnitude of the illegal movements is currently unknown.
- Should a movement ban be considered and considering the complexity of the taxonomy as well as the lack of evidences related to all the species, a movement ban at the level of taxonomic order is likely to be both more effective and more feasible.
- The feasibility of a movement ban mainly depends on the import volumes.
- The effectiveness of a movement ban is mainly dependent on the import volumes, possibility of Bsal to remain viable outside susceptible/tolerant species (e.g. fomites, travel boxes, etc.), and the capacity to limit illegal movements.

ToR 3: Validity, reliability and robustness of the available diagnostic methods for the detection of Bsa

- The clinical symptoms linked to Bsal are characterised by marked skin ulcerations, but they are in general variable and not pathognomonic. Infection can be present in a salamander without any clinical symptoms.

- Although no specific study has been conducted with the aim of estimating the sensitivity and specificity of the histological examination, these parameters are likely to be relatively low. In any case, considering the similarity of the lesions induced by Bd and Bsal and of the pathogens themselves, the histological examination as such cannot be used as a confirmatory diagnostic test.
- Isolation in live culture, considering the time-consuming procedure to grow *Batrachochytrium* fungi, is not a preferred method of Bsal diagnostics. The DSe has not been assessed, but is estimated to be very low. Characterisation of cultured fungi is further dependent on genetic identification.
- The validation of the duplex real-time PCR has completed the first two validation stages, but not the third stage as foreseen in the OIE guidelines.
- Based on the estimates that current data lead to, it results that: (i) the test is not suitable for prevalence studies; (ii) the test could fail in detecting infected animals; and (iii) the test could still fit for a freedom from disease framework, although a safe approach would imply a considerably high sample size. These considerations are based on and due to the statistically limited sample size used in the validation process and do not necessarily reflect the actual performance of the test.

ToR 4: Possible alternative methods and feasible risk mitigation measures to ensure safe international and EU trade of salamanders and their products

- The mitigation measures that have been identified as relevant and feasible for ensuring safe international and EU trade of salamanders are: (i) quarantining salamanders, (ii) enacting legislation that requires testing of the animals to demonstrate freedom from Bsal, before movement can take place, (iii) restricting salamander movements, (iv) tracking all traded species, (v) hygienic procedures/biosecurity measures before and during movements, and (vi) increasing public awareness.
- Regarding quarantining salamanders, it is possible to estimate the sample size needed in order to assess, with a 95% confidence, if the consignment is free from Bsal, based on the number of animals included in the consignment and on the DSe of the test used. Assuming a worst-case scenario with a DSe equal to 0.5, (i) the size of the consignment cannot be smaller than 432, (ii) all animals should be tested, and (iii) all test results should be negative. Different parameters and scenarios can be set according to the needs.
- Animal by-products derived from salamanders heat treated at 25°C for at least 10 days are not considered relevant for the spread of Bsal to the salamander populations in the EU, as Bsal is not able to survive in such temperatures.
- Desiccated animal by-products derived from salamanders are not considered relevant for the spread of Bsal to the salamander populations in the EU, as the fungus is dependent on water and desiccation is fatal to all life stages.

Recommendations

ToR 1: Assessment of the potential of Bsal to affect the health of wild and kept salamanders in the Union

- A meta-analysis, across all available infection experiments, should be carried out to overcome sample size limitations in individual infection experiments and for species susceptibility comparisons.
- The association between Bsal and mortality in *S. salamandra* and other European species should be further assessed in additional studies to address the small sample sizes and the uncertainty regarding the representativeness of the animal subpopulations used in the experiments, e.g. with robust field studies and disease surveillance.
- Further experimental studies are required to quantify differences in species susceptibility to Bsal leading to robust assessments regarding potential host/risk status of all potentially traded/moved salamanders. This is particularly necessary in the case of a species-specific movement ban being considered.

- The relative contribution of Bsal to population trends requires further investigation, e.g. a monitoring of field data on salamander populations and Bsal prevalence, through time.

ToR 2: Effectiveness and feasibility of a movement (including intra-EU trade and introduction from non-EU countries) ban of traded salamanders, including both Asian and non-Asian species

- Data collection on the movement of all caudata species into EU and between EU countries should be put in place at species level.
- Effectiveness and feasibility assessments of a movement ban need to be considered in the context of other actions that could potentially mitigate the threat of Bsal in the EU.

ToR 3: Validity, reliability and robustness of the available diagnostic methods for the detection of Bsa

- Formal validation of available detection assays, particularly duplex real-time PCR, would be beneficial in view of their official recognition.
- More scientific studies should be conducted in order to reduce the uncertainty around the key performance parameters of the duplex real-time PCR.

ToR 4: Possible alternative methods and feasible risk mitigation measures to ensure safe international and EU trade of salamanders and their products

- An assessment of the relative feasibility and effectiveness of available methods or combinations of methods to ensure safe movements and trade of salamanders should be carried out.

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Glossary and Abbreviations

AHAW	Animal Health and Welfare
AHL	Animal Health Law
Assurance colony	A captive population of a critically endangered species that is being carefully managed and bred for long-term survival of the species.
Area of occupancy	The area within its 'extent of occurrence' which is occupied by a taxon, excluding cases of vagrancy.
ARMI	Amphibian Research and Monitoring Initiative
Bd	<i>Batrachochytrium dendrobatidis</i>
Bsal	<i>Batrachochytrium salamandrivorans</i>
CITES	Convention on International Trade in Endangered Species
DSe	Test diagnostic sensitivity
DSp	Test diagnostic specificity
ELR	Extensive Literature Review
Extent of occurrence	The area contained within the shortest continuous imaginary boundary which can be drawn to encompass all the known, inferred or projected sites of present occurrence of a taxon, excluding cases of vagrancy.
Extinction	the loss of an entire species
Extirpation	the loss of a population
ITS	internal transcribed spacer
IUCN	International Union for Conservation of Nature
LEMIS	Law Enforcement Management Information System
MSs	Member States
Newts	Representatives of one group within the family Salamandridae (subfamily Pleurodelinae). For taxonomic consistency, the use in this report of the term 'salamanders' is inclusive of 'newts'
NTP-OHAT	National Toxicology Program-Office of Health Assessment and Translation
PCR	polymerase chain reaction
ToRs	Terms of Reference
TRACES	Trade Control and Expert System. Is the European Commission's multilingual online management tool for all sanitary requirements on intra-EU trade and importation of animals, semen and embryo, food, feed and plants
UNEP-WCMC	United Nations Environment Programme – World Conservation Monitoring Centre; based in Cambridge, UK.
USFWS	US Fish and Wildlife Service
USGS	US Geological Survey
WG	Working group

Appendix A – CITES and EU wildlife trade legal framework²⁵

The CITES Convention

The Convention on International Trade in Endangered Species (CITES)⁶ regulates international trade in more than 35,000 animal and plant species. There are 932 species listed in CITES Appendix I, 34,419 species listed in Appendix II and 147 species listed in Appendix III (35,597 species in total).

Appendix I includes species threatened with extinction, for which trade must be subject to stricter regulation, and can only be authorised in exceptional circumstances for specimens of wild origin. Commercial trade in wild taken specimens of Appendix I-listed species is generally not allowed.

Appendix II includes species that are not necessarily now threatened with extinction but may become so unless trade is strictly regulated. Appendix II further lists so-called 'look-alike species', which are controlled because of their similarity in appearance to other regulated species, thereby facilitating more effective control.

Appendix III contains species that are subject to regulation within the jurisdiction of a CITES Party and for which the co-operation of other CITES Parties is needed to prevent or restrict their exploitation.

CITES has 183 Parties and each Party reports on a yearly basis on its import and export of CITES-listed species to the CITES Secretariat. These trade data are available publicly.

The Regulation (EC) No 338/97⁷

The implementation of CITES within the EU is governed by EU regulations, which are directly applicable in the Member States. The EU Wildlife Trade Regulations and CITES cover trade in all specimens, whether alive or dead, including parts and derivatives, from animal and plant species listed in the Annexes/Appendices. The term 'trade' encompasses not only trade in a commercial sense but also, for example, imports and (re)-exports for personal use. The EU Regulation is stricter than the CITES Convention and it lists more species in its Annexes, than is the number of those listed in CITES Appendices.

The species covered by Regulation (EC) No 338/97 are listed in four Annexes. In some cases, entire genera or families are listed.

Annex A of the EU Regulation lists 1,203 species and subspecies. It contains all CITES Appendix I-listed species and other species (listed in CITES Appendix II, III, or non-CITES-listed) that are, or may be, in EU or international demand for trade and which are either threatened with extinction or are so rare that any trade would imperil its survival in the wild. Annex A lists also some look-alike species if this is considered to be essential for the effective protection of the species listed in Annex A, in order to exclude commercial trade in the entire genus or species (e.g. for reasons related to control/enforcement). This Annex also lists most of the species native to the EU that are protected by the EU legislation (the so-called 'Birds' and 'Habitats' Directives).

Commercial trade from, to and within the EU is, as a general rule, prohibited for wild specimens of species listed in Annex A. External trade to and from the EU is governed by provisions comparable to those applicable to species listed in Appendix I under CITES.

Annex B contains 36,478 species, which are CITES Appendix II-listed species (if they are not already included in Annex A), and other species (CITES Appendix III-listed, non-CITES-listed) subject to levels of international trade that might not be compatible with the survival of populations in certain countries, or with the maintenance of its total population at a level that is consistent with its role in the ecosystem. Annex B also lists some look-alike species, whose listing is considered essential for the effective control of trade in other species listed in Annex A or B. In addition, Annex B also lists species known to pose an ecological threat to species that are indigenous to the EU.

Documentation is required for the import, export and (re-)export of specimens of Annex B-listed species into/from the EU. EU rules on import of Annex B-listed species are stricter than under CITES as import permits are required (in addition to export permits) for the import of such specimens into the EU.

Annex C contains 150 species, which are CITES Appendix III-listed species that are not already included in Annex A or B.

Species listed in Annex C do not require an import permit. Imports can take place on the basis of a CITES export permit, a (re-)export certificate, or a certificate of origin, together with an import notification (the import notification is not a document required under CITES and is therefore a stricter

²⁵ Based on the Reference Guide to the European Union Wildlife Trade Regulations, January 2015, Traffic, http://ec.europa.eu/environment/cites/pdf/referenceguide_en.pdf

EU measure). The (re-)export of specimens of Annex C-listed species from the EU requires an export permit or re-export certificate.

Annex D contains 169 species, which are non-CITES-listed species that are not listed in Annexes A to C which are imported into the EU in such numbers as to warrant monitoring, and Appendix III-listed species for which EU Member States have entered a reservation (there are currently three of these (and four subspecies)).

Annex D lists species that do not have a CITES equivalent. Imports of specimens of Annex D-listed species require an import notification. The Annex D monitoring system is intended to allow the early detection of possible conservation concerns to the species listed.

Caudata species currently listed in the CITES Appendices and/or in EU Wildlife Trade Regulations-Annexes

Thirty-seven species of Caudata are listed under the CITES Appendices (I, II or III) and/or the Annexes (A, B, C or D) of the EU Wildlife Trade Regulations. They pertain to five families: Ambystomatidae, Cryptobranchidae, Hynobiidae, Plethodontidae and Salamandridae (see Table A.1). Some of species are listed under the species-specific name (e.g.: *Ambystoma mexicanum* is listed as such, see last column of the table), whereas others are listed under a general species name (e.g.: the eleven species of *Paramesotriton* are all listed under '*Paramesotriton* spp.').

Table A.1: Caudata species currently listed in the CITES Appendices^(a) and/or in EU Wildlife Trade Regulations-Annexes

Family	Species	EU Wildlife Trade Regulations-Annex	CITES Appendix	Listed under
Ambystomatidae	<i>Ambystoma dumerilii</i>	B	II	<i>Ambystoma dumerilii</i>
Ambystomatidae	<i>Ambystoma mexicanum</i>	B	II	<i>Ambystoma mexicanum</i>
Cryptobranchidae	<i>Andrias davidianus</i>	A	I	<i>Andrias</i> spp.
Cryptobranchidae	<i>Andrias japonicus</i>	A	I	<i>Andrias</i> spp.
Cryptobranchidae	<i>Cryptobranchus alleganiensis</i>	C	III	<i>Cryptobranchus alleganiensis</i>
Hynobiidae	<i>Hynobius amjiensis</i>	C	III	<i>Hynobius amjiensis</i>
Hynobiidae	<i>Ranodon sibiricus</i>	D	NC*	<i>Ranodon sibiricus</i>
Plethodontidae	<i>Bolitoglossa dofleini</i>	D	NC	<i>Bolitoglossa dofleini</i>
Salamandridae	<i>Cynops ensicauda</i>	D	NC	<i>Cynops ensicauda</i>
Salamandridae	<i>Echinotriton andersoni</i>	D	NC	<i>Echinotriton andersoni</i>
Salamandridae	<i>Laotriton laoensis</i>	D	NC	<i>Laotriton laoensis</i>
Salamandridae	<i>Neurergus kaiseri</i>	A	I	<i>Neurergus kaiseri</i>
Salamandridae	<i>Paramesotriton caudopunctatus</i>	D	NC	<i>Paramesotriton</i> spp.
Salamandridae	<i>Paramesotriton chinensis</i>	D	NC	<i>Paramesotriton</i> spp.
Salamandridae	<i>Paramesotriton deloustali</i>	D	NC	<i>Paramesotriton</i> spp.
Salamandridae	<i>Paramesotriton fuzhongensis</i>	D	NC	<i>Paramesotriton</i> spp.
Salamandridae	<i>Paramesotriton guanxiensis</i>	D	NC	<i>Paramesotriton</i> spp.
Salamandridae	<i>Paramesotriton hongkongensis</i>	D	II	<i>Paramesotriton</i> spp.
Salamandridae	<i>Paramesotriton labiatus</i>	D	NC	<i>Paramesotriton</i> spp.
Salamandridae	<i>Paramesotriton longliensis</i>	D	NC	<i>Paramesotriton</i> spp.
Salamandridae	<i>Paramesotriton maolanensis</i>	D	NC	<i>Paramesotriton</i> spp.
Salamandridae	<i>Paramesotriton yunwuensis</i>	D	NC	<i>Paramesotriton</i> spp.
Salamandridae	<i>Paramesotriton zhijinensis</i>	D	NC	<i>Paramesotriton</i> spp.
Salamandridae	<i>Salamandra algira</i>	D	III	<i>Salamandra algira</i>
Salamandridae	<i>Tylototriton asperrimus</i>	D	NC	<i>Tylototriton</i> spp.
Salamandridae	<i>Tylototriton broadoridgus</i>	D	NC	<i>Tylototriton</i> spp.
Salamandridae	<i>Tylototriton dabienicus</i>	D	NC	<i>Tylototriton</i> spp.
Salamandridae	<i>Tylototriton hainanensis</i>	D	NC	<i>Tylototriton</i> spp.
Salamandridae	<i>Tylototriton kweichowensis</i>	D	NC	<i>Tylototriton</i> spp.
Salamandridae	<i>Tylototriton lizhengchangi</i>	D	NC	<i>Tylototriton</i> spp.

Family	Species	EU Wildlife Trade Regulations-Annex	CITES Appendix	Listed under
Salamandridae	<i>Tylototriton notialis</i>	D	NC	<i>Tylototriton</i> spp.
Salamandridae	<i>Tylototriton pseudoverrucosus</i>	D	NC	<i>Tylototriton</i> spp.
Salamandridae	<i>Tylototriton taliangensis</i>	D	NC	<i>Tylototriton</i> spp.
Salamandridae	<i>Tylototriton verrucosus</i>	D	NC	<i>Tylototriton</i> spp.
Salamandridae	<i>Tylototriton vietnamensis</i>	D	NC	<i>Tylototriton</i> spp.
Salamandridae	<i>Tylototriton wenxianensis</i>	D	NC	<i>Tylototriton</i> spp.
Salamandridae	<i>Tylototriton yangi</i>	D	NC	<i>Tylototriton</i> spp.

*NC: not classified.

(a): From <https://cites.org/eng/app/appendices.php> – Downloaded on January 2017.

Appendix B – Extensive Literature Search

B.1. Sources of information included in the search:

A) Bibliographic databases

1) Web of Science, encompassing the following databases:

- Web of Science™ Core Collection (1975–present)
- BIOSIS Citation IndexSM (1926–present)
- CABI: CAB Abstracts[®] (1910–present)
- Chinese Science Citation DatabaseSM (1989–present)
- Current Contents Connect[®] (1998–present)
- Data Citation IndexSM (1900–present)
- FSTA[®] – the food science resource (1969–present)
- KCI-Korean Journal Database (1980–present)
- Russian Science Citation Index (2005–present)
- MEDLINE[®] (1950–present)
- SciELO Citation Index (1997–present)
- Zoological Record[®] (1864–present)

2) Scopus (1960–present) (Scopus site)

3) PubMed (1946–present) (PubMed site)

B) Search engines

1) Invasive Species Compendium (<http://www.cabi.org/isc/>): bibliographic and datasheet search

2) Google Scholar: in order to facilitate the treatment of the results, Google Scholar will be searched via Publish and Perish (<http://www.harzing.com/resources/publish-or-perish>)

3) OpenAIRE (<https://www.openaire.eu/>)

4) Worldwidescience (<http://www.worldwidescience.org>): papers and public papers.

B.1.1. Search strings used in bibliographic databases

The search strings were designed to retrieve relevant documents to '*Batrachochytrium salamandrivorans*'. The genus name, *Batrachochytrium*, was not included as an independent term in the search strings in an attempt to maximise the precision of the searches, since it will retrieve publications on '*Batrachochytrium dendrobatidis*' that are non-relevant for the purpose of this report.

1) Web of Science (all databases)

Date of the search 19/10/2016

Set	Query	Results
#1	TS=(((Bsal OR Bs) AND (Urodela* OR salamander* OR salamandr* OR Newt\$ OR amphibia*)) OR salamandrivorans) Timespan=All years Search language=Auto	87

2) Scopus

Date of the search 19/10/2016

Set	Query	Results
#1	TITLE-ABS-KEY (((bsal OR bs) AND (urodela* OR salamand* OR newt\$ OR amphibia*)) OR salamandrivorans)	32

3) Pubmed

Date of the search 19/10/2016

Set	Query	Results
#3	Search (((("Urodela"[Mesh] OR urodela*[tiab] OR salamandr*[tiab] OR salamander*[tiab] OR newt[tiab] OR newts[tiab] OR amphibia*[tiab]) AND (Bsal[tiab] OR Bs[tiab]))) OR salamandrivorans[tiab])	20
#2	Search salamandrivorans[tiab] Sort by: PublicationDate	15
#1	Search (("Urodela"[Mesh] OR urodela*[tiab] OR salamandr*[tiab] OR salamander*[tiab] OR newt[tiab] OR newts[tiab] OR amphibia*[tiab]) AND (Bsal[tiab] OR Bs[tiab])) Sort by: PublicationDate	10

B.1.2. Search strings used in search engines1) Invasive Species Compendium. Advanced bibliographic search (<http://www.cabi.org/isc/>)

Date of the search 19/10/2016

Query	Results
(((Bsal OR Bs) AND (urodela* OR salamander* OR salamandr* OR newt* OR amphibia*))) OR (salamandrivorans) Item Types: Abstract, CABI Book Chapter Info, CABI Book Chapter Info, Full Text, Library	8

2) Invasive Species Compendium. Advanced datasheet search (<http://www.cabi.org/isc/>)

Date of the search 19/10/2016

Query	Results
(((Bsal OR Bs) AND (urodela* OR salamander* OR salamandr* OR newt* OR amphibia*))) OR (salamandrivorans)	2

3) Google Scholar (via Publish and Perish)

Date of the search 20/10/2016

Query	Results
All the words: Salamandrivorans	242

4) OpenAIRE (<https://www.openaire.eu/>)

Date of the search 19/10/2016

Query	Results
Salamandrivorans	18

5) Worldwidescience (<http://www.worldwidescience.org>)^{26,27}

Date of the search 20/10/2016

Query	Results
Salamandrivorans	119

²⁶ The source of information CSIR Research Space (South Africa) was excluded from the search, since problems with the retrieval system for this specific source were detected. The source was searched using their own website (<http://researchspace.csir.co.za/dspace/>) and no relevant results were found.

²⁷ The results of Worldwidescience were limited to papers and public access publication.

B.2. Refinement of literature search results

A total of 528 records resulted from the initial searches and were exported to an EndNote library. Duplicates were first removed by resource; the 444 resulting records were de-duplicated across all resources. This yielded 311 records which were distributed in three EndNote libraries/excel files:

- 1) Bsal bibliographic database, including the results for Web of Science (all databases), PubMed and Scopus and after de-duplication: 90 records.
- 2) Bsal Google scholar, including the results for Google scholar and after de-duplication: 188 records.
- 3) Bsal other search engines, including the results for OpenAIRE, Worldwidescience, and Invasive Species Compendium: 33 records.

Titles and abstracts were screened for relevance and to remove additional duplicates. Full text publications were screened if title and abstract did not allow assessing the relevance of a paper. The screening was performed by one reviewer, with support by a second reviewer in case of doubt about the relevance of a paper. Thirteen duplicates were removed, and 67 records resulted as pertaining to *Batrachochytrium salamandrivorans* (26 from the Bsal bibliographic database, 35 from the Bsal Google scholar library and 6 from the Bsal other search engines).

The full text of the publications was assessed if they were peer-reviewed primary studies or grey literature relevant for Bsal. A total number of 33 records (30 publication and 3 supplementing materials) were considered to be relevant to the search question (19 records from the Bsal bibliographic database, 11 from the Bsal Google scholar library and 3 from the Bsal other search engines). For each resulting paper, it was indicated for which ToR (one or more) it contained relevant information (see Table B.2).

Another full text screening was carried out by *ad hoc* experts and some of the publications were not considered relevant or to provide additional value to address the question.

As part of addressing ToR 1, a range of eligibility criteria were established in order to identify studies that allow assessing if a causal relationship between Bsal and disease/mortality in salamanders exists. These were

- target population salamanders or newts;
- experimental study with a control group;
- description of route of exposure;
- description of levels/doses used for the infection;
- description of duration of the exposure and the follow-up;
- description of temperature;
- description of observed mortality or of population decline or other adverse effect on health;
- description of the place the experiment was carried out;
- paper written in English (at least the abstract);

The 15 papers relevant for ToR 1 were screened for their eligibility against these criteria. Two studies that reported experimental studies fulfilling all eligibility criteria were identified. These were included in the critical appraisal.

An overview of the numbers of the records that resulted from each step of the ELR is reported in Table B.1.

Table B.1: Overview of the number of results of the ELR

Initial search	Database		Initial count	Post de-duplication within the same database results
19/10/2016	Web of Science (All databases)		87	81
19/10/2016	Pubmed		20	20
19/10/2016	Scopus		32	32
Web searching	Resource		Initial count	Post de-duplication within the same resource results
19/10/2016	Invasive Species Compendium. Bibliographic search		8	8
19/10/2016	Invasive Species Compendium. Datasheet search		2	2
19/10/2016	Google Scholar		242	235
19/10/2016	OpenAIRE		18	17
20/10/2016	Worldwidescience		119	49
		Total	528	444
				Number of records
			After de-duplication among all the resources	311
			After screening of titles and abstracts to identify additional duplicates and relevant literature	67
			After full texts screening to identify relevant literature	33
				Number of publications
			After full texts screening to identify relevant literature	30
			After full texts screening to identify relevant literature for ToR 1	15
			After full texts screening to identify relevant literature for causal relationship between Bsal and disease/mortality in salamanders	2

Table B.2: List of relevant publications resulting from the Extensive Literature Review by ToR

ID	Citation References	Relevance to:			
		ToR 1 (impact on health)	ToR 2 (trade)	ToR 3 (diagnostic tests)	ToR 4 (mitigation measures)
1	Auliya et al. (2016)		X		
2	Bales et al. (2015)	X		X	
3	Blooi et al. (2013)			X	
4	Blooi et al. (2015a)	X			X
5	Blooi et al. (2015b)	X			X
6	Brink (2015)				X
7	Cunningham et al. (2015)	X			
8	Gimeno et al. (2015)	X			
9	Gleason et al. (2014)				X
10	Grant et al. (2016a)				X
11	Grant et al. (2016b)				X
12	Gray et al. (2015)		X		X
13	James et al. (2015)				X

Citation		Relevance to:			
ID	References	ToR 1 (impact on health)	ToR 2 (trade)	ToR 3 (diagnostic tests)	ToR 4 (mitigation measures)
14	Martel et al. (2013)*	X		X	
15	Martel et al. (2014)*	X			
16	Muletz et al. (2014)	X			
17	Parrott et al. (2016)	X		X	
18	Rollins-Smith (2016)	X			
19	Rowley et al. (2016)		X		
20	Sabino-Pinto et al. (2015)	X			
21	Sanchez et al. (2016)	X			
22	Semlitsch et al. (2017)				X
23	Spitzen-van der Sluijs et al. (2015)		X		
24	Spitzen-van der Sluijs et al. (2016)	X			
25	Stephen (2015)				X
26	Van Rooij et al. (2015)	X			X
27	Wombwell (2014)		X		
28	Woodhams et al. (2016)				X
29	Yap et al. (2015)		X		X
30	Zhu et al. (2014)	X	X	X	

*: Studies that were identified allow assessing if a causal relationship between Bsal and disease/mortality in salamanders exists.

Appendix C – Caudata in Europe

Table C.1: Number of native species of salamanders in EU Member States (<http://amphibiaweb.org>, data downloaded 15 December 2016)

Country	Number of salamander species	Country	Number of salamander species
Austria	7	Italy	19
Belgium	5	Latvia	2
Bulgaria	6	Lithuania	2
Croatia	7	Luxembourg	5
Cyprus	0	Malta	0
Czech Republic	8	Netherlands	6
Denmark	3	Poland	5
Estonia	2	Portugal	7
Finland	2	Romania	6
France	13	Slovakia	6
Germany	6	Slovenia	5
Greece	7	Spain	10
Hungary	5	Sweden	2
Ireland	1	United Kingdom	4

Table C.2: The biodiversity of Caudata present in European countries, their conservation status and observed population change(*)

Family	Genus species	Article 17 of the Habitats Directive Period 2007–2012	IUCN Red List conservation status	Population trend IUCN
Proteidae	<i>Proteus anguinus</i>	U1	VU	Decreasing
Plethodontidae	<i>Speleomantes ambrosii</i>	FV	NT	Stable
	<i>Speleomantes flavus</i>	FV	VU	Stable
	<i>Speleomantes genei</i>	U1	VU	Decreasing
	<i>Speleomantes imperialis</i>	FV	NT	Stable
	<i>Speleomantes strinatii</i>	FV	NT	Stable
	<i>Speleomantes supramontis</i>	FV	EN	Decreasing
	<i>Speleomantes sarrabusensis</i>	Not assessed	VU	Stable
	<i>Speleomantes italicus</i>	Not assessed	NT	Stable
Salamandridae	<i>Calotriton arnoldi</i>	Not assessed	CR	Decreasing
	<i>Calotriton asper</i>	U1, XX	NT	Decreasing
	<i>Euproctus montanus</i>	FV	LC	Stable
	<i>Euproctus platycephalus</i>	U1	EN	Decreasing
	<i>Chioglossa lusitanica</i>	FV, U1	VU	Decreasing
	<i>Ichthyosaura alpestris</i>	Not assessed	LC	Decreasing
	<i>Lissotriton boscai</i>	Not assessed	LC	Stable
	<i>Lissotriton helveticus</i>	Not assessed	LC	Stable
	<i>Lissotriton italicus</i>	FV	LC	Decreasing
	<i>Lissotriton montandoni</i>	U2, XX	LC	Decreasing
	<i>Lissotriton vulgaris</i>	Not assessed	LC	Stable
	<i>Lyciasalamandra helverseni</i>	Not assessed	VU	Stable
	<i>Lyciasalamandra luschani</i>	Not assessed	VU	Stable
	<i>Pleurodeles waltl</i>	Not assessed	NT	Decreasing
	<i>Salamandra atra</i>	FV, U1	LC	Decreasing
<i>Salamandra atra aurorae</i>	U2	CR subspecies	Decreasing	

Family	Genus species	Article 17 of the Habitats Directive Period 2007–2012	IUCN Red List conservation status	Population trend IUCN
	<i>Salamandra corsica</i>	Not assessed	LC	Stable
	<i>Salamandra lanzai</i>	U1	VU	Stable
	<i>Salamandra salamandra</i>	Not assessed	LC	Decreasing
	<i>Salamandrina perspicillata</i>	Not assessed	LC	Stable
	<i>Salamandrina terdigitata</i>	FV	LC	Stable
	<i>Triturus carnifex</i>	U1, U2	LC	Decreasing
	<i>Triturus cristatus</i>	U1, U2, XX	LC	Decreasing
	<i>Triturus dobrogicus</i>	U1, U2, XX	NT	Decreasing
	<i>Triturus ivanbureschi</i>	Not assessed	Not assessed	Not assessed
	<i>Triturus karelinii</i>	FV, U1, U2	LC	Decreasing
	<i>Triturus macedonicus</i>	U1, U2	Not assessed	
	<i>Triturus marmoratus</i>	U2, XX	LC	Decreasing
	<i>Triturus pygmaeus</i>	Not assessed	NT	Decreasing

(*): CRITICALLY ENDANGERED (CR): extremely high risk of extinction in the wild;
 ENDANGERED (EN): very high risk of extinction in the wild;
 VULNERABLE (VU): high risk of extinction in the wild;
 NEAR THREATENED (NT): close to qualifying for or is likely to qualify for a threatened category in the near future; LEAST CONCERN (LC): Widespread and abundant taxa. Reporting under Article 17: 'Favourable Conservation Status' FV the species can be expected to prosper without any change to existing management or policies; 'Unfavourable-Inadequate' U1 a change in management or policy is required to return the species to favourable status, but there is no danger of extinction in the foreseeable future; 'Unfavourable-Bad' U2 is for species in serious danger of becoming extinct (at least regionally); 'Unknown' (XX) insufficient information is available to allow an assessment. The potential risk of Bsal to salamanders was not yet included in these categorisations.

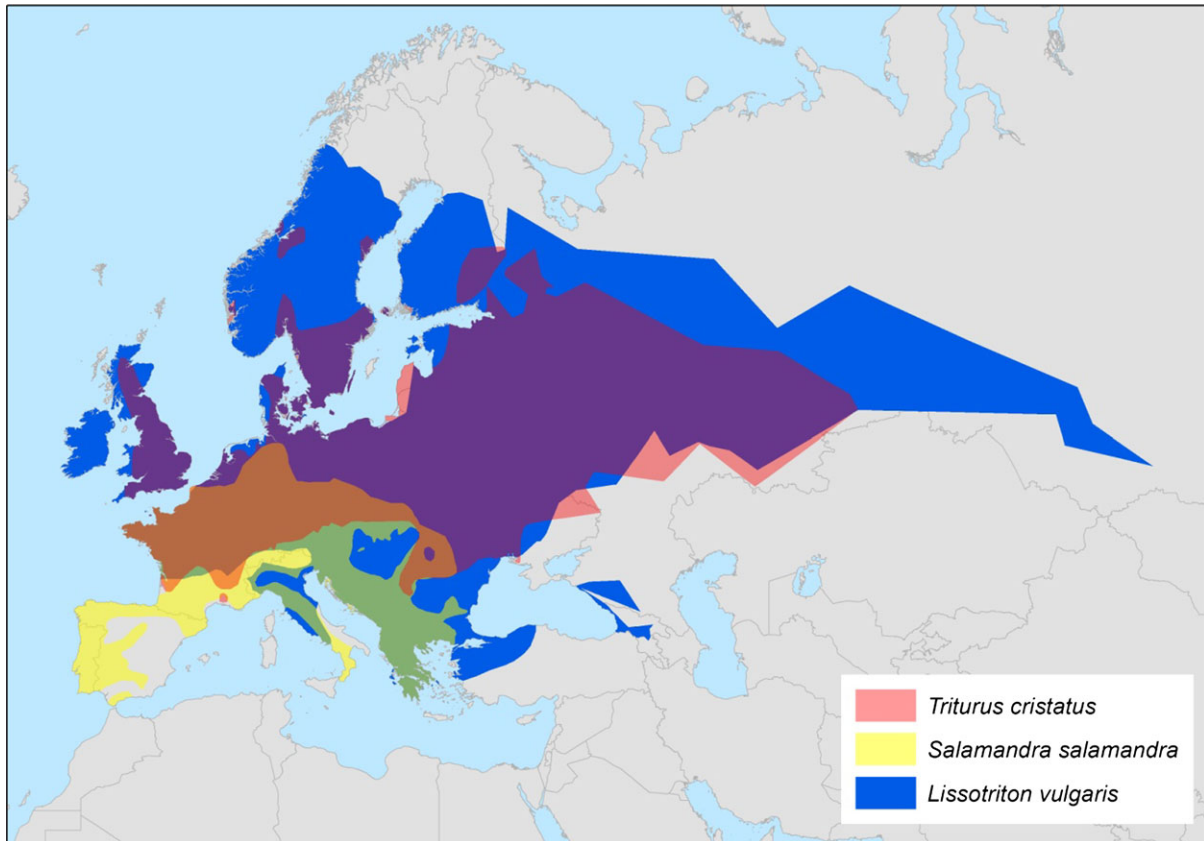


Figure C.1: Distribution of the three most important species in Europe: *Triturus cristatus*²⁸ (in red), *Salamandra salamandra*²⁹ (in yellow) and *Lissotriton vulgaris*³⁰ (in blue). Green, orange and purple are given by the overlapping of the areas

²⁸ Jan Willem Arntzen, Sergius Kuzmin, Robert Jehle, Trevor Beebee, David Tarkhnishvili, Vladimir Ishchenko, Natalia Ananjeva, Nikolai Orlov, Boris Tuniyev, Mathieu Denoël, Per Nyström, Brandon Anthony, Benedikt Schmidt, Agnieszka Ogradowczyk. 2009. *Triturus cristatus*. The IUCN Red List of Threatened Species 2009: e.T22212A9365894. <https://doi.org/10.2305/IUCN.UK.2009.RLTS.T22212A9365894.en>

²⁹ Sergius Kuzmin, Theodore Papenfuss, Max Sparreboom, Ismail H. Ugurtas, Steven Anderson, Trevor Beebee, Mathieu Denoël, Franco Andreone, Brandon Anthony, Benedikt Schmidt, Agnieszka Ogradowczyk, Maria Ogielska, Jaime Bosch, David Tarkhnishvili, Vladimir Ishchenko. 2009. *Salamandra salamandra*. The IUCN Red List of Threatened Species 2009: e.T59467A11928351. <https://doi.org/10.2305/IUCN.UK.2009.RLTS.T59467A11928351.en>

³⁰ Jan Willem Arntzen, Sergius Kuzmin, Trevor Beebee, Theodore Papenfuss, Max Sparreboom, Ismail H. Ugurtas, Steven Anderson, Brandon Anthony, Franco Andreone, David Tarkhnishvili, Vladimir Ishchenko, Natalia Ananjeva, Nikolai Orlov, Boris Tuniyev. 2009. *Lissotriton vulgaris*. The IUCN Red List of Threatened Species 2009: e.T59481A11932252. <https://doi.org/10.2305/IUCN.UK.2009.RLTS.T59481A11932252.en>

Appendix D – Results of critical appraisal of infection experiments

Table D.1: Overview of the results of the critical appraisal of experiment study 1 (Martel et al., 2013)

Critical appraisal criteria	Experimental study 1	RoB (H = High, L = Low)	Expected bias direction and magnitude (only for HRoB)
Randomisation	Yes	L	
Allocation concealment	Yes	L	
Experimental conditions identical across study group	Yes	L	
Research personnel and human subjects blinded to the study group	Yes	L	
Outcome data complete without attrition or exclusion from the analysis	Yes	L	
Appropriate exposure characterisation	Yes	L	
Appropriate outcome assessment	Yes	L	
All measured outcomes reported	Main reported	L	
Biologically relevant effect identified	Yes	L	
Appropriate sample size	Very small sample size (5 animals per group)	H	In case of presence of the disease, the effect could be overestimated, in case of absence, the effect could be underestimated
Appropriate statistical methods used to summarise data (e.g. model)	Not reported	L	
Appropriate method to treat missing data	N/A		

Table D.2: Overview of the results of the critical appraisal of experiment study 2 (Martel et al., 2013)

Critical appraisal criteria	Experimental study 2	RoB (H = High, L = Low)	Expected bias direction and magnitude (only for HRoB)
Randomisation	Yes	L	
Allocation concealment	Yes	L	
Experimental conditions identical across study group	Yes	L	
Research personnel and human subjects blinded to the study group	Yes	L	
Outcome data complete without attrition or exclusion from the analysis	Yes	L	
Appropriate exposure characterisation	Yes	L	
Appropriate outcome assessment	Yes	L	
All measured outcomes reported	Main reported	L	
Biologically relevant effect identified	Yes	L	
Appropriate sample size	Very small sample size (3 animals per group)	H	In case of presence of the disease, the effect could be overestimated, in case of absence, the effect could be underestimated
Appropriate statistical methods used to summarise data (e.g. model)	Not reported	L	
Appropriate method to treat missing data	N/A		

Table D.3: Overview of the results of the critical appraisal of experiment study 3 (Martel et al., 2013)

Critical appraisal criteria	Experimental study 3	RoB (H = High, L = Low)	Expected bias direction and magnitude (only for HRoB)
Randomisation	Yes	L	
Allocation concealment	Yes	L	

Critical appraisal criteria	Experimental study 3	RoB (H = High, L = Low)	Expected bias direction and magnitude (only for HRoB)
Experimental conditions identical across study group	Yes	L	
Research personnel and human subjects blinded to the study group	Yes	L	
Outcome data complete without attrition or exclusion from the analysis	Yes	L	
Appropriate exposure characterisation	Yes	L	
Appropriate outcome assessment	Yes	L	
All measured outcomes reported	Main reported	L	
Biologically relevant effect identified	Yes	L	
Appropriate sample size	Very small sample size (5 animals per group)	H	In case of presence of the disease, the effect could be overestimated, in case of absence, the effect could be underestimated
Appropriate statistical methods used to summarise data (e.g. model)	Not reported	L	
Appropriate method to treat missing data	N/A		

Table D.4: Overview of the results of the critical appraisal of experiment study 4 (Martel et al., 2014)

Critical appraisal criteria	Experimental study 4	RoB (H = High, L = Low)	Expected bias direction and magnitude (only for HRoB)
Randomisation	Yes	L	
Allocation concealment	Yes	L	
Experimental conditions identical across study group	Yes	L	
Research personnel and human subjects blinded to the study group	Yes	L	
Outcome data complete without attrition or exclusion from the analysis	Yes	L	
Appropriate exposure characterisation	Yes	L	
Appropriate outcome assessment	Yes	L	
All measured outcomes reported	Main reported	L	
Biologically relevant effect identified	Yes	L	
Appropriate sample size	Very small sample size	H	In case of presence of the disease, the effect could be overestimated, in case of absence, the effect could be underestimated
Appropriate statistical methods used to summarise data (e.g. model)	Not reported	L	
Appropriate method to treat missing data	N/A		

Table D.5: Overview of the results of the critical appraisal of experiment study 5 (Martel et al., 2014)

Critical appraisal criteria	Experimental study 5	RoB (H = High, L = Low)	Expected bias direction and magnitude (only for HRoB)
Randomisation	Yes	L	
Allocation concealment	Yes	L	
Experimental conditions identical across study group	Yes	L	
Research personnel and human subjects blinded to the study group	Yes	L	
Outcome data complete without attrition or exclusion from the analysis	Yes	L	

Critical appraisal criteria	Experimental study 5	RoB (H = High, L = Low)	Expected bias direction and magnitude (only for HRoB)
Appropriate exposure characterisation	Yes	L	
Appropriate outcome assessment	Yes	L	
All measured outcomes reported	Main reported	L	
Biologically relevant effect identified	Yes	L	
Appropriate sample size	Very small sample size	H	In case of presence of the disease, the effect could be overestimated, in case of absence, the effect could be underestimated
Appropriate statistical methods used to summarise data (e.g. model)	Not reported	L	
Appropriate method to treat missing data	N/A		

Table D.6: Overview of the results of the critical appraisal of experiment study 6 (Martel et al., 2014)

Critical appraisal criteria	Experimental study 6	RoB (H = High, L = Low)	Expected bias direction and magnitude (only for HRoB)
Randomisation	Yes	L	
Allocation concealment	Yes	L	
Experimental conditions identical across study group	Yes	L	
Research personnel and human subjects blinded to the study group	Yes	L	
Outcome data complete without attrition or exclusion from the analysis	Yes	L	
Appropriate exposure characterisation	Yes	L	
Appropriate outcome assessment	Yes	L	
All measured outcomes reported	Main reported	L	
Biologically relevant effect identified	Yes	L	
Appropriate sample size	Very small sample size	H	In case of presence of the disease, the effect could be overestimated, in case of absence, the effect could be underestimated
Appropriate statistical methods used to summarise data (e.g. model)	Not reported	L	
Appropriate method to treat missing data	N/A		

Table D.7: Overview of the results of the critical appraisal of experiment study 7 (Martel et al., 2014)

Critical appraisal criteria	Experimental study 7	RoB (H = High, L = Low)	Expected bias direction and magnitude (only for HRoB)
Randomisation	Yes	L	
Allocation concealment	Yes	L	
Experimental conditions identical across study group	Yes	L	
Research personnel and human subjects blinded to the study group	Yes	L	
Outcome data complete without attrition or exclusion from the analysis	Yes	L	
Appropriate exposure characterisation	Yes	L	
Appropriate outcome assessment	Yes	L	
All measured outcomes reported	Only Bsal loads as determined by real-time PCR are reported, the outcomes of the daily clinical monitoring are not reported	L	

Critical appraisal criteria	Experimental study 7	RoB (H = High, L = Low)	Expected bias direction and magnitude (only for HRoB)
Biologically relevant effect identified	Yes	L	
Appropriate sample size	Very small sample size	H	In case of presence of the disease, the effect could be overestimated, in case of absence, the effect could be underestimated
Appropriate statistical methods used to summarise data (e.g. model)	Not reported	L	
Appropriate method to treat missing data	N/A		

Appendix E – Bsal detection in EU salamanders

Table E.1: Summary of the available data on the ability of Bsal to infect salamanders (wild or captive individuals) and the observed results of infection

Data from experimental exposures				
Category	Family	Species	Source	
Resistant	Ambystomatidae	<i>Ambystoma maculatum</i>	Martel et al. (2014)	
	Ambystomatidae	<i>Ambystoma opacum</i>	Martel et al. (2014)	
	Hynobiidae	<i>Hynobius retardatus</i>	Martel et al. (2014)	
	Hynobiidae	<i>Pachyhynobius shangchengensis</i>	Martel et al. (2014)	
	Plethodontidae	<i>Gyrinophilus porphyriticus</i>	Martel et al. (2014)	
	Plethodontidae	<i>Plethodon glutinosus</i>	Martel et al. (2014)	
	Salamandridae	<i>Lissotriton helveticus*</i>	Martel et al. (2014)	
Tolerant	Hynobiidae	<i>Salamandrella keyserlingii</i>	Martel et al. (2014)	
	Sirenidae	<i>Siren intermedia</i>	Martel et al. (2014)	
Susceptible	Salamandridae	<i>Cynops cyanurus</i>	Martel et al. (2014)	
	Salamandridae	<i>Cynops pyrrhogaster</i>	Martel et al. (2014)	
	Salamandridae	<i>Paramesotriton deloustali</i>	Martel et al. (2014)	
Lethally susceptible	Plethodontidae	<i>Hydromantes strinatii*</i>	Martel et al. (2014)	
	Salamandridae	<i>Euproctus platycephalus*</i>	Martel et al. (2014)	
	Salamandridae	<i>Ichthyosaura alpestris*</i>	Martel et al. (2014)	
	Salamandridae	<i>Lissotriton italicus*</i>	Martel et al. (2014)	
	Salamandridae	<i>Neurergus crocatus</i>	Martel et al., 2014	
	Salamandridae	<i>Notophthalmus viridescens</i>	Martel et al. (2014)	
	Salamandridae	<i>Pleurodeles waltl*</i>	Martel et al. (2014)	
	Salamandridae	<i>Salamandra salamandra*</i>	Martel et al. (2014)	
	Salamandridae	<i>Salamandrina perspicillata*</i>	Martel et al. (2014)	
	Salamandridae	<i>Taricha granulosa</i>	Martel et al. (2014)	
	Salamandridae	<i>Triturus cristatus*</i>	Martel et al. (2014)	
	Salamandridae	<i>Tylotriton wenxianensis</i>	Martel et al. (2014)	
	Bsal infection in wild, captive or museum specimens			
Hynobiidae		<i>Hynobius nebulosus</i>	Martel et al. (2014)	
Hynobiidae		<i>Onychodactylus japonicas</i>	Martel et al. (2014)	
Hynobiidae		<i>Salamandrella keyserlingii</i>	Martel et al. (2014)	
Salamandridae		<i>Cynops ensicauda</i>	Martel et al. (2014)	
Salamandridae		<i>Cynops pyrrhogaster</i>	Martel et al. (2014)	
Salamandridae		<i>Paramesotriton deloustali</i>	Martel et al. (2014)	
Salamandridae		<i>Tylotriton uyenoii</i>	Martel et al. (2014)	
Salamandridae		<i>Tylotriton vietnamensis</i>	Martel et al. (2014)	
Salamandridae		<i>Tylotriton zieglerei</i>	Martel et al. (2014)	
Linked with observed mortality		Salamandridae	<i>Ichthyosaura alpestris*</i>	Spitzen-van der Sluijs et al. (2016)
Linked with observed mortality		Salamandridae	<i>Lissotriton vulgaris*</i>	Spitzen-van der Sluijs et al. (2016)
Linked with observed mortality		Salamandridae	<i>Salamandra salamandra*</i>	Spitzen-van der Sluijs et al. (2016)
Linked with observed mortality	Salamandridae	<i>Salamandra algira</i>	Sabino-Pinto et al. (2015)	
Linked with observed mortality	Salamandridae	<i>Salamandra salamandra</i> (10 subspecies)*	Sabino-Pinto et al. (2015)	

Data from experimental exposures

Category	Family	Species	Source
Linked with observed mortality	Plethodontidae	<i>Speleomantes</i> spp.*	Cunningham et al. (2015)

*Species present in wild populations in EU.



Figure E.1: The present known distribution of Bsal (14 detection sites, see black dots in the figure) in the Netherlands, Belgium and Germany covers approximately 10,000 km²

Appendix F – Evaluations made for diagnostic test evaluation of the real-time quantitative PCR³¹

Table F.1: Data related to the performance of the real-time PCR (Bloom et al., 2013)

Feature	Subfeature	Description	Evaluation method and results
1	Specificity of the primer (analytical specificity)	–	Melting curve analysis All Bsal-positive samples generated a single peak in the SYBR green real-time PCR melting curve analysis. Gel electrophoresis of the PCR product of <i>B. salamandrivorans</i> -positive samples and positive controls always generated a single DNA and at the expected size. Negative samples and negative controls did not generate a peak in the melting curve analysis or generate a visible band in gel electrophoresis, indicating that no nonspecific binding of the primers occurred
2	Primer and probe concentration	First simplex assay	The lowest concentrations that yielded the highest $\Delta R_n^{(a)}$
3	Amplification efficiency	Intra-assay Inter-assay	$E = 10^{-(1/\text{slope})} - 1 = 95.7$ 96.0
4	Linear standard curve	Intra-assay Inter-assay Simplex vs duplex	Coefficient of determination (r^2) = 0.999 0.997 0.997
5	Precision	Intra-assay variability Inter-assay variability	Mean coefficient of variation = 0.39 ± 0.48 Mean coefficient of variation = 0.98 ± 0.27
6	LoD (analytical sensitivity)	–	0.1 GE per PCR
7	Mean quantification cycle	BSA ^(b) vs No BSA	$C_q^{(c)} (0.1 \text{ GE}) = 36.18 \pm 0.23 \text{ (BSA)}/36.18 \pm 0.16 \text{ (without BSA)}$

(a): Defined as the R_n value (the fluorescence emission of the reporter dye, normalised to the background fluorescence) of a reaction mixture containing all the reaction components (including the template) minus the R_n value of an unreacted negative control.

(b): Bovine serum albumin.

(c): Quantification cycle.

³¹Data extracted from Bloom et al. (2013).

Table F.2: Criteria as described in the OIE Manual of Diagnostic Tests for Aquatic Animals (2016) and related evidence of fulfilment as reported in Blooi et al. (2013)

Section	Subsection	Point	Addressed/ passed	Comment
1.2 – Assay development – the experimental studies	–	–	Not relevant (preliminary steps)	–
2.1 – Stage 1 Analytical performance characteristics	2.1.1 – Repeatability	–	Yes	See Table F.1 – Points 3, 4 and 5
	2.1.2 – Analytical specificity	–	Yes	See Table F.1 – Point 1
	2.1.3 – Analytical sensitivity	–	Yes	See Table F.1 – Point 6
	2.1.4 – Analytical accuracy of adjunct tests or procedures	–	Not relevant	–
2.2 – Stage 2 Diagnostic performance of the assay	–	–	–	–
	2.2.1 – Reference animal populations	2.2.1.1 – Negative reference samples	Satisfied by point 2.2.3	The 51 samples from Merelbeke were not confirmed to be negative by any other diagnostic methodology (or data are not reported). The simple fact of having being collected in an area that 'have never had the disease in question' is not applicable in this case as this is an emerging disease
		2.2.1.2 – Positive reference samples	Satisfied by point 2.2.3	The 41 samples collected from a declining population were not confirmed to be positive by any other diagnostic methodology (or data not reported). In addition, it is reported that the individuals were 'apparently healthy'
	2.2.2 – Samples from animals of unknown status	–	Satisfied by point 2.2.3	Latent class analysis not possible as the samples from the field did not undergo any other test
	2.2.3 – Experimentally infected or vaccinated reference animals	–	Yes	Infection experiment 5 positive individuals (truly positive) 5 controls (truly negative)
	2.2.4 – Cut-off (threshold) determination	–	Yes	Coincides with LoD
2.2.5 – Calculation of DSe and DSp based on test results of reference samples	–	–	Yes	Not explicitly reported, but data are available (see point 2.2.3) The statistically small sample size provides a wide confidence interval. DSe and DSp estimates as provided in Section 3.4.3 of this report can be considered as preliminary estimates, fulfilling criterion 2.2.6
	2.2.6 – Provisional assay recognition	–	Yes ^(a)	DSe and DSp can be estimated, although the confidence interval is wide (see Section 3.4.3 of this report) The confidence in the estimates can be enhanced by additional experimental results

Section	Subsection	Point	Addressed/ passed	Comment
2.3 – Stage 3 Reproducibility and augmented repeatability estimates	2.3.1 – Reproducibility	–	No	The same samples were not tested in any other laboratory using the same methodology
	2.3.2 – Designation of a validated assay	–	Not relevant at this stage	–

(a): Provisional recognition does not imply acceptance by the OIE. It does, however, recognise an informed decision of authorities at local, state, national or international levels of their conditional approval of a partially validated assay (OIE, 2016; footnote¹³).

Appendix G – Trade data

EU-28 trade data from CITES Trade Database³²

Official volumes of traded animals, (both direct and indirect imports) from 2005 to 2015, and relevant ancillary data (e.g. origin, purpose of the trade, importing and exporting countries, etc.), were provided by the UNEP-WCMC³³ for the species of Caudata listed under CITES Appendices and Annexes of the EU Wildlife Trade Regulations.

Thirty-seven species of Caudata are listed under the CITES Appendices and/or the Annexes of the EU Wildlife Trade Regulations and pertain to five families: Ambystomatidae, Cryptobranchidae, Hynobiidae, Plethodontidae and Salamandridae. Three of these families resulted containing at least one species with individuals that have shown to be tolerant to Bsal (Hynobiidae, Plethodontidae, Salamandridae, see Section 3.3.3), whereas none of the species of the fourth family with individuals that have shown to be Bsal tolerant (Sirenidae) are listed in the CITES Appendices and/or EU wildlife Trade Regulations-Annexes (for details on the species of Caudata currently listed, see Appendix A).

Trade data are available for 16 of the listed species pertaining to four of the five families (no reported trade in the species of Plethodontidae); trade to EU-28 was reported by importing countries for 10 (pertaining to Ambystomatidae, Cryptobranchidae, and Salamandridae) of the 16 species (see Figure G.2 and Tables G.2–G.4 in this Appendix).

Overview of the import into EU-28 of caudata species listed in CITES Appendices and/or EU Wildlife Trade Regulations in the years 2005–2015

From the available data, the estimated number of imports into EU-28 of caudata species listed in CITES Appendices and/or EU Wildlife Trade Regulations-Annexes is very heterogeneous along the years and no relevant time trend (not increasing or declining trends) can be assessed. However, in general it can be said that most of the imports (movements and individuals imported) occurred between 2009 and 2013 (see Figure G.1).

Estimates of imports into EU-28 per year (2005–2015) (log scale)

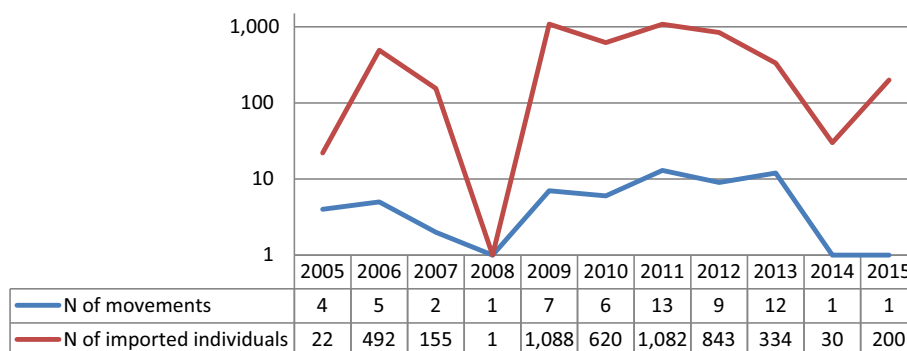


Figure G.1: Graph of the imports (movements and imported individuals) into EU-28 (years 2005–2015) of caudata species listed in CITES Appendices and/or EU Wildlife Trade Regulations-Annexes (data reported by the importers)

Between 2005 and 2015, as reported by the importers, it was estimated that 61 importing movements occurred into EU-28 for a total number of 4,867 individuals (including live, specimens, skeletons and bodies) of species listed in CITES Appendices and/or Annexes of EU Wildlife Trade Regulations (see Table G.2). Of these estimates, 22 importing movements and 1,119 individuals regarded species listed in CITES Appendices.

The species listed in CITES Appendices and/or EU Wildlife Trade Regulations that were reported as main imported ones were: *Paramesotriton chinensis*, *Ambystoma mexicanum*, *Tylototriton Kweichowensis*, *Paramesotriton labiatus* and *Tylototriton asperrimus* (see Figure G.2).

³² CITES trade statistics reported in this section derived from the data of the CITES Trade Database, UNEP-WCMC, Cambridge, UK, which have been downloaded on the 25 January 2017. It has to be noted that the CITES Trade Data is not intended for the use of counting individual shipments/movements, therefore the figures reported in this section should be considered as estimates.

³³ UNEP-WCMC is the specialist biodiversity assessment arm of the United Nations Environment Programme (UNEP) based in Cambridge, UK. www.unep-wcmc.org

In the specific case of *Paramesotriton deloustali*, which is listed in Annex D of the EU Wildlife Trade Regulations and has been categorised as 'susceptible' carrier of Bsal (Martel et al., 2014), there has been no reported trade between 2005 and 2015 into EU-28. In addition, it was no find any evidence of this species for sale within the European Union in a short survey of EU websites conducted in 2016 by UNEP-WCMC.

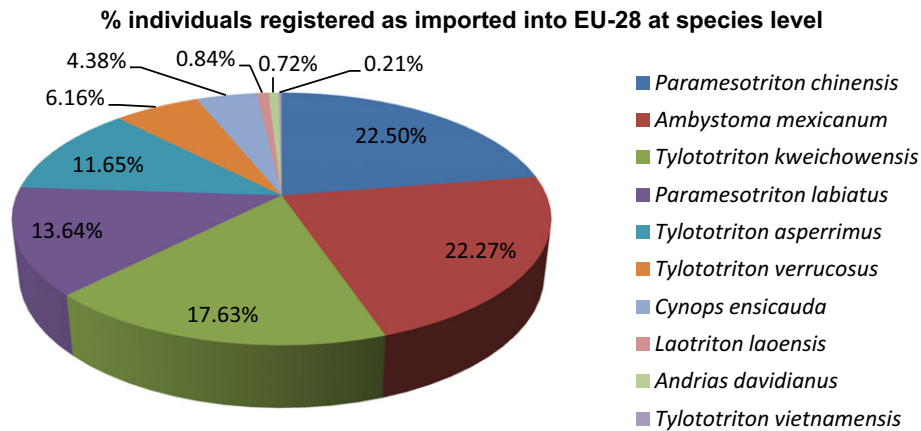


Figure G.2: Caudata species listed in CITES Appendices and/or Annexes of the EU Wildlife Trade Regulations and imported into EU-28 between 2005 and 2015 (data reported by the importers)

The countries of EU-28 that reported the majority of individuals imported were Germany (3,269 individuals - all 'live'), the Czech Republic and Spain (Figure G.3).

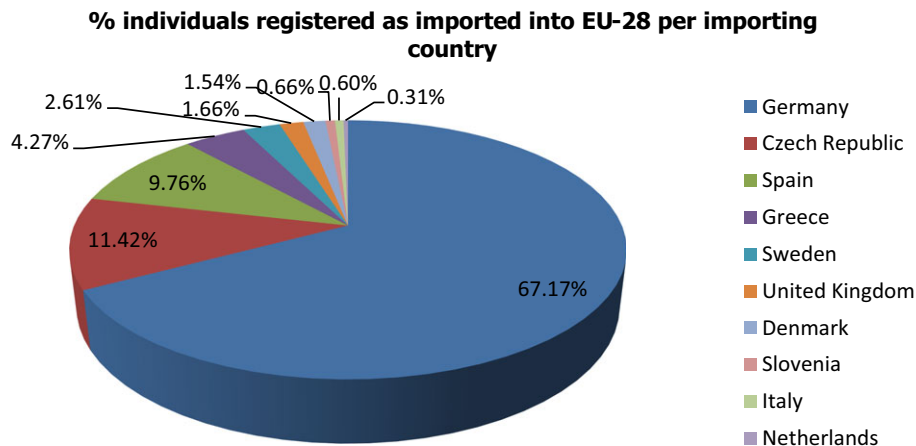


Figure G.3: Countries of EU-28 that reported most imports (individuals) of caudata species listed in CITES Appendices and/or Annexes of the EU Wildlife Trade Regulations between 2005 and 2015 (data reported by the importers)

The 'origin' of the importing movements may vary; the main providers of caudata species listed in CITES Appendices and/or Annexes of the EU Wildlife Trade Regulations between 2005 and 2015 to EU-28 are estimated to be China, United States, Hong Kong-SAR and Japan (see Figure G.4).

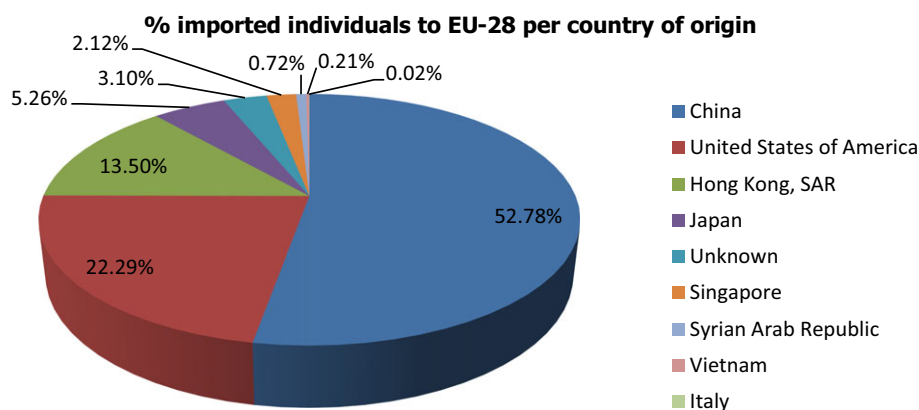


Figure G.4: Countries of origin that were reported to provide the majority of individuals of caudata species listed in CITES Appendices and/or Annexes of the EU Wildlife Trade Regulations into EU-28 between 2005 and 2015 (data reported by the importers)³⁴

From the available data, it is estimated that, between 2005 and 2015 individuals were imported into EU-28 mainly for commercial purposes, primarily from unknown sources. The main known sources of the imported animals are estimated to be 'Animals bred in captivity' and 'Specimens taken from the wild' for both direct and indirect trade, whereas 'Specimens taken from the wild' are estimated to be mainly subjected to indirect trade. A low percentage of specimens taken from the wild was reported for the direct trade (see Table G.1).

Table G.1: Reported purposes and sources (% of individuals) of the direct and indirect importing trades of caudata species listed in CITES Appendices and/or Annexes of the EU Wildlife Trade Regulations into EU-28 between 2005 and 2015 (data reported by the importers)

	Direct trade (Tot = 4,022 individuals)	Indirect trade (Tot = 845 individuals)
Purpose		
Commercial	59.45%	95.62%
Scientific purpose	26.53%	–
Not specified	13.65%	–
Medical (including biomedical research)	0.37%	–
Zoo	–	4.14%
Educational	–	0.12%
Circus or travelling exhibition	–	0.12%
Source		
Unknown	54.60%	54.20%
Specimens taken from the wild	2.98%	23.67%
Animals bred in captivity	28.77%	22.01%
Not specified	13.65%	–
Preconvention specimens	–	0.12%

³⁴ Italy is here reported because the import was indirect: one individual (live) was exported for 'circus or travelling exhibition' from Italy (country of origin) to Montenegro, and from Montenegro (exporting country), it was exported back to Italy (EU-28 importing Country). For more details, see Table G.4.

Table G.2: Data on imports (importing movements and individuals as reported by the importers) of caudata species listed in CITES Appendices and/or Annexes of the EU Wildlife Trade Regulations that were estimated to occur into EU-28 (year 2005–2015)

Year	Ambystomatidae		Cryptobranchidae		Salamandridae		Total Sum of movements	Total Sum of imported individuals
	Sum of movements	Sum of imported individuals	Sum of movements	Sum of imported individuals	Sum of movements	Sum of imported individuals		
2005	4	22					4	22
2006	5	492 (22 bodies) ^(a)					5	492
2007	2	155 (105 specimens)					2	155
2008	1	1 (skeleton)					1	1
2009	3	224			4	864	7	1,088
2010	2	95			4	525	6	620
2011	1	30			12	1,052	13	1,082
2012					9	843	9	843
2013	2	35	1	35	9	264	12	334
2014	1	30					1	30
2015					1	200	1	200
Grand Total	21	1,084	1	35	39	3,748	62	4,867

Source: CITES Trade Database, UNEP-WCMC; Cambridge, UK, downloaded on 25 January 2017.

(a): In brackets the number of individuals else than 'live' is specified; however, it is already included in the relevant 'sum of imported individuals'.

Table G.3: Direct imports of individuals (including live, specimens, skeletons and bodies) of caudata species listed in CITES Appendices and/or Annexes of the EU Wildlife Trade Regulations into EU-28 between 2005 and 2015 (data reported by the importers). No direct imports of species listed under CITES Appendices and/or Annexes of the EU Wildlife Trade Regulations were registered to EU-28 in the years 2008 and 2015

Family	Taxon	Importing country ISO Code	Exporting country ISO Code	Term	Purpose	Source	Year								Grand Total		
							2005	2006	2007	2009	2010	2011	2012	2013		2014	
Ambystomatidae	<i>Ambystoma mexicanum</i>	DK	US	Live	S	C				75						75	
		DE	US	Live	S	C		460		15	20					495	
		GR	US	Live	S	C			208							208	
		NL	US	Live	M	C							15			15	
		SI	US	Live	S	C		22	10							32	
		ES	US	Live	S	C				50						50	
		SE	US	Bodies	S	C			22							22	
		SE	US	Specimens	S	C				105						105	
		UK	US	Live	S	C						30			20	30	80
		DE	JP	Live	T	U					100	75			38		213
Salamandridae	<i>Cynops ensicauda</i>	DE	JP	Live	T	U								41		41	
		DE	JP	Live	T	U											
	<i>Paramesotriton chinensis</i>	CZ	CN	Live	-	-				62						62	
		CZ	HK-SAR	Live	-	-						128				128	
	<i>Paramesotriton labiatus</i>	DE	CN	Live	T	U				600	152			50		802	
		DE	HK-SAR	Live	T	U				100						100	
	<i>Paramesotriton labiatus</i>	DE	US	Live	T	U								3		3	
		CZ	CN	Live	-	-				102						102	
		CZ	HK-SAR	Live	-	-						159				159	
		DE	CN	Live	T	U					150					150	
	IT	SG	SG	Live	-	-						28				28	
																	ES

Family	Taxon	Importing country ISO Code	Exporting country ISO Code	Term	Purpose	Source	Year								Grand Total
							2005	2006	2007	2009	2010	2011	2012	2013	
	<i>Tylotriton asperrimus</i>	CZ	HK-SAR	Live	-	-					70				70
		DE	CN	Live	T	U				125					125
		DE	CN	Live	T	W						120			120
		DE	JP	Live	T	U							2		2
	<i>Tylotriton kweichowensis</i>	DE	CN	Live	T	U					350				350
		DE	HK-SAR	Live	T	U			200						200
	<i>Tylotriton verrucosus</i>	DE	CN	Live	T	U					200				200
		DE	VN	Live	T	U							10		10
Grand Total							22	492	1,087	470	1,082	385	299	30	4,022

Source: CITES Trade Database, UNEP-WCMC; Cambridge, UK, downloaded on 25 January 2017.

Purpose codes: M = Medical (including biomedical research), S = Scientific, T = Commercial.

Source codes: C = Animals bred in captivity in accordance with Resolution Conf. 10.16 (Rev.), as well as parts and derivatives thereof, exported under the provisions of Article VII, paragraph 5, of the Convention, U = Source unknown, W = Specimens taken from the wild.

Table G.4: Indirect imports of individuals (including live, specimens, skeletons and bodies) of caudata species listed in CITES Appendices and/or Annexes of the EU Wildlife Trade Regulations into EU-28 between 2005 and 2015 (data reported by the importers). No indirect imports of species listed under CITES Appendices and/or Annexes of the EU Wildlife Trade Regulations were registered to EU-28 in the years 2005, 2006, 2007, 2011 and 2014

Family	Taxon	Importing country ISO Code	Country of Origin ISO Code	Exporting country ISO Code	Term	Purpose	Source	Year					Grand Total	
								2008	2009	2010	2012	2013		2015
Ambystomatidae	<i>Ambystoma mexicanum</i>	IT	IT	ME	Live	Q	C		1					1
		UK	Unknown	CA	Skeletons	E	O	1						1
Cryptobranchidae	<i>Andrias davidianus</i>	CZ	SY	KR	Live	Z	C				35			35
Salamandridae	<i>Paramesotriton labiatus</i>	ES	Unknown	SG	Live	T	C			150				150
	<i>Tylotriton asperimus</i>	DE	CN	CN	Live	T	U				50			50
		ES	CN	HK-SAR	Live	T	W						200	200
	<i>Tylotriton kweichowensis</i>	DE	CN	CN	Live	T	U				308			308
	<i>Tylotriton verrucosus</i>	DE	CN	CN	Live	T	U				100			100
Grand Total								1	1	150	458	35	200	845

Source: CITES Trade Database, UNEP-WCMC; Cambridge, UK, downloaded on 25 January 2017.

Purpose codes: E = Educational, Q = Circus or travelling exhibition, T = Commercial, Z = Zoo.

Source codes: C = Animals bred in captivity in accordance with Resolution Conf. 10.16 (Rev.), as well as parts and derivatives thereof, exported under the provisions of Article VII, paragraph 5, of the Convention, O = Preconvention specimens, U = Source unknown, W = Specimens taken from the wild.

US trade data from CITES Trade Database³²

The import of Caudata into the US regards the species listed in CITES Appendices which pertain to the families Ambystomatidae, Cryptobranchidae and Hynobiidae. None of the Salamandridae listed under CITES Appendices were reported to be traded into the US between 2005 and 2015 (see Table G.5).

From the CITES Trade database, the estimated imports of Caudata listed in CITES Appendices into the US are: 31 importing movements for a total of 1,536 imported individuals (including eggs, specimens, live, skeletons, bodies and meat (data reported by the importers) – see Table G.5). From these data, the estimated imports of Caudata listed in CITES Appendices into EU-28 represent about 70% of the import of individuals in the US (see above).

Also in the case of US, the import is very heterogeneous along the years and no relevant time trend (not increasing or declining trends) can be assessed. However, in general, it can be said that the most of the imports (movements and individuals imported) occurred between 2009 and 2015 (with the exclusion of 2012 and 2014) (see Figure G.5).

Estimates of imports into the US per year (2005–2015) (log scale)

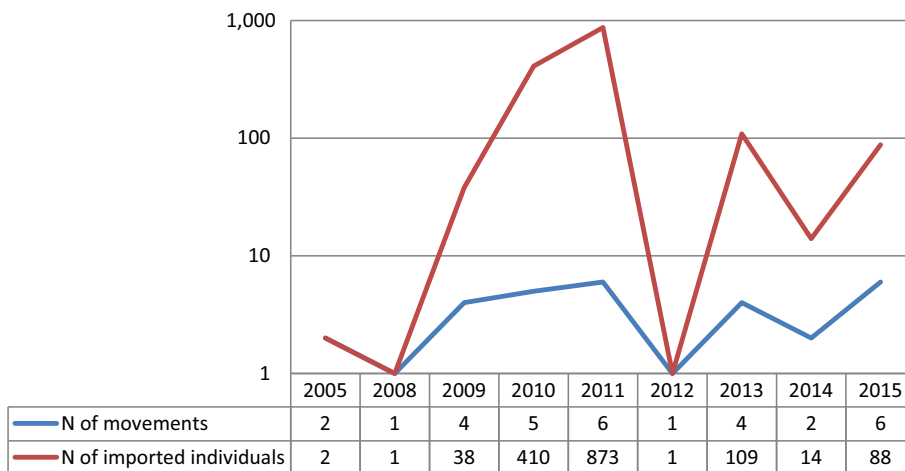


Figure G.5: Graph of the imports (movements and imported individuals) in the US (years 2005–2015) of caudata species listed in CITES Appendices (data reported by the importers). No imports of species listed under CITES Appendices were registered to US in the years 2006 and 2007

Table G.5: Data on imports (importing movements and individuals as reported by the importers) of caudata species listed in CITES Appendices that were estimated to occur into US (years 2005–2015). No imports of species listed under CITES Appendices were registered to US in the years 2006 and 2007

Year	Ambystomatidae		Cryptobranchidae		Hynobiidae		Total Sum of movements	Total Sum of imported individuals
	Sum of movements	Sum of imported individuals	Sum of movements	Sum of imported individuals	Sum of movements	Sum of imported individuals		
2005	1	1	1	1 (skeleton) ^(a)			2	2
2008			1	1 (specimen)			1	1
2009	2	31 (1 skeleton)	2	7 (1 specimen)			4	38
2010	5	410					5	410
2011	5	872	1	1			6	873
2012	1	1 (specimen)					1	1
2013	3	108	1	1 (meat)			4	109
2014	2	14 (10 bodies)					2	14
2015	1	80 (eggs(live))	4	7 (4 specimens)	1	1 (specimen)	6	88
Grand Total	20	1,517	10	18	1	1	31	1,536

Source: CITES Trade Database, UNEP-WCMC; Cambridge, UK, downloaded on 25 January 2017.

(a): In brackets the number of individuals else than 'live' is specified; however, it is already included in the relevant 'sum of imported individuals'.

Appendix H – Monitoring Bsal and its hosts

Amphibian populations are monitored through different survey methods depending on the habitat and the species. Some field methods just record detection/non-detection data but outcomes can be modelled for detection probability, accounting for unequal effort and other sources of observation error (Grant et al., 2016b).

Bsal should be monitored through qualified diagnostic methods and establishing standardised laboratory practices. Early detection of Bsal in wild populations can only be accomplished through a robust, well-designed surveillance of natural populations of salamanders and newts that responds to morbidity and mortality events reported through a well-organised database and communication network (Grant et al., 2016a). In the EU, Bsal monitoring is taking place in several countries, but is not homogeneous. Belgium, the Netherlands and Germany have already started surveillance of the pathogen in field, salamander population monitoring and public awareness campaigns. Bsal was detected in trade in the UK; subsequently, public awareness and biosecurity guidance have been promoted by the Institute of Zoology and the Government, but no specific surveillance scheme has been set up. In the Czech Republic, attempts to detect Bsal in wild and captive salamanders have started in Prague in autumn 2015. The remaining countries that provided response on the Recommendation No. 176 (2015) have not reported any specific surveillance activities in place (see Follow-up of Recommendation No. 176 (2015) on the prevention and control of the *Batrachochytrium salamandrivorans* chytrid fungus).³⁵

Populations are considered in decline when they have a decreasing abundance trend, as established by robust monitoring. Extirpation typically refers to the loss of a population. Extinction refers to the loss of an entire species. Formal classification of extinction can take decades, as confidence in complete absence can take a long time to build (e.g. the IUCN's definition is that a 'A taxon is Extinct when there is no reasonable doubt that the last individual has died. A taxon is presumed Extinct when exhaustive surveys in known and/or expected habitat, at appropriate times (diurnal, seasonal, annual), throughout its historic range have failed to record an individual. Surveys should be over a time frame appropriate to the taxon's life cycle and life form').³⁶

The closely related pathogen, Bd, has caused or contributed to declines, extirpation and extinction of amphibians. Bsal has so far not been implicated in extinctions but population declines of over 96% have been observed associated with Bsal presence, Het Bunderbos, the Netherlands) (see Section 3.3).

Extirpation of populations becomes increasingly likely as populations dwindle – small population sizes are more vulnerable to direct and indirect threats as well as stochastic processes (e.g. extreme weather events). Species often become more vulnerable to extinction when there are fewer populations/with reduced populations sizes/reduced range sizes. Hence, declines and population extirpations contribute to extinction risk. An example of an extinction risk framework is the IUCN Red List. Degree of extinction risk is assessed using multiple criteria.³⁶

Population declines, extirpations of species and extinctions can have downstream impacts on the broader ecosystems in which they occur, e.g. by altering species interactions such as predator–prey dynamics. This can include impacts on ecosystems services (e.g., Hocking and Babbitt, 2014).

³⁵ <https://wcd.coe.int/ViewDoc.jsp?p=&id=2438037&Site=&direct=true>

³⁶ http://www.iucnredlist.org/static/categories_criteria_3_1#definitions

Appendix I – Immediate disease management actions

Treatment of infected consignments with appropriate fungicides to clear infection could be considered (regarded as potentially feasible at wholesalers, if suitable biosecure housing demonstrated and post-treatment disease free status verified; see paragraph below and Wombwell, 2014) as well as euthanasia and proper disposal of infected consignments (Wombwell, 2014), compulsory notification and pathogen screening of all domestic imports (< 5 animals imported by individual collectors). This would require individuals entering the EU with amphibians for their personal collection, to inform import authorities, and impose an obligation to have animals screened at a point of import (Wombwell, 2014). Other actions could be restricting site-level access, decontaminating a site and removal of amphibians from the site. However, containment management responses is regarded as rather ineffective in preventing the long-term spread of Bsal if not acted on with urgency and decisiveness on first identification (Grant et al., 2016a). Developing a management plan and timeline for actions prior to, or as soon as possible after, pathogen arrival is crucial because early action is often considerably more cost effective than the same approach taken later (Langwig et al., 2015).

Deployment of Bsal zoospore removal methods do not exist for live animals, only antifungal treatment removes infectious Bsal particles from an animal. However, this measure might not be commercially viable due to the cost this would incur.

Environmental manipulation

Options suggested in a review on Bd include shallow warm water for tadpoles, decrease shading to create open basking sites for adults and metamorphs, artificial heat sources (all life stages), exclude (Bd) reservoir host species, introduce (Bd) inhibitors (salts, fungicides) and alter water flow or pond drying regime (as reviewed in Scheele et al., 2014). Environmental disinfection was tested in specific conditions against Bd with positive results (Bosch et al., 2015); however, so far this has only been successful in one study system despite multiple efforts globally over a significant period, and it is unknown how long site level disinfection will persist given the high invasiveness of Bd. The longer term prospects and costs of maintaining freedom from disease are thus currently impossible to estimate, but significant investment of resources and ongoing effort is likely. Furthermore, specific environmental conditions may be required for environmental disinfection to be successful (e.g. pond systems that can be contained versus stream systems that are more complex). These options are very likely applicable to Bsal, but results may vary due to biological differences of the host species (e.g. some salamander species may not thrive in the site if water temperatures are elevated by environmental manipulation).

Treatments

These include heating (to 25°C; Blooi et al., 2015a), antifungals (e.g. Blooi et al., 2015b), bioaugmentation with commensal bacteria and probiotics (Muletz et al., 2012; Bletz et al., 2013; Woodhams et al., 2016) among others (Kueneman et al., 2016). Hudson et al. (2016) tested the *in-situ* treatment of individual mountain chicken frogs (*Leptodactylus fallax*) using the antifungal drug, itraconazole. Multistate mark-recapture analysis showed increased probability of survival and loss of Bd infection for treated frogs compared to untreated animals. There was evidence of a prophylactic effect of treatment as, during the treatment period, infection probability was lower for treated animals than untreated animals. While long-term, post-treatment increase in survival was not observed, a deterministic population model estimated antifungal treatment would extend time to extinction of the population from 49 to 124 weeks, an approximated 60% increase. *In-situ* treatment of individuals could, therefore, be a useful short-term measure to augment other conservation actions for amphibian species threatened by chytridiomycosis or to facilitate population survival during periods of high disease risk. Specifically, in a caudata individual (fire salamander; *S. salamandra*), 'topical treatment with voriconazole or itraconazole alone (12.5 µg/mL and 0.6 µg/mL, respectively) or in combination with polymyxin E (2,000 IU/mL) at an ambient temperature of 15°C during 10 days decreased fungal loads but did not clear Bd infections. However, topical treatment of Bd infected animals with a combination of polymyxin E (2,000 IU/mL) and voriconazole (12.5 µg/mL) at an ambient temperature of 20°C resulted in clearance of Bd infections' (Blooi et al., 2015b). Probiotics and other means of altering amphibian skin microbiota ('bioaugmentation of the skin mucosome') have been tested as a treatment against Bd. However, probiotics may not provide a generalised solution for amphibian fungal diseases (Woodhams et al., 2016). Again, long-term viability of maintaining healthy wild populations/preventing declines with individual treatments is unknown, but resources and effort are expected to be substantial and ongoing.

Long-term management actions

This could include selective breeding for tolerance or resistance and prophylactic treatments (mentioned in Grant et al., 2016a), as well as translocations to clean sites and other *ex-situ* conservation options (Scheele et al., 2014). These could be seen as measures to create a Bsal-free environment.

In order to better cope with wildlife diseases, it has been suggested to set up national and international wildlife health strategies able to provide a full spectrum of wildlife population health activities (Stephen, 2015). International institutions should work with government agencies and experts in disease ecology and animal trade to implement an international effort for surveillance, research and management actions in an adaptive management framework for effective wildlife disease intervention (Langwig et al., 2015; Yap et al., 2015). Specifically for Bsal, a National Task Force has been set up for the US, composed of a technical advisory committee and seven working groups. Initiatives to reduce the risk of Bsal introduction are also taking place in Canada, and a need for a trilateral approach including Mexico, has been proposed for North America (Gray et al., 2015).

Movements of wild salamanders within the EU MSs in conservation efforts (e.g. population translocations in cases of site destruction) are a specific case. They can adopt mitigation measures that are not likely to be applicable, in terms of efforts, for trade; e.g. strict biosecurity measures, testing all individuals for infection and, in case of detection of a pathogen, obligatory treatment (antifungal or heat).

Treatment of water used in transport

Water that came in contact with infected salamanders can contain infectious zoospores of Bsal. The possible treatments that were tested against Bd are likely to be able to inactivate zoospores of Bsal. These treatments include: (i) the use of various disinfectants (Virkon™ per mL, benzalkonium chloride, didecyl dimethyl ammonium chloride, sodium hypochloride), or (ii) the use of elevated temperature. All waste water that came in contact with infected salamanders, or with those of unclear infection status, is to be treated before disposal. The concentration of active substances and duration of treatment should follow the recommendations of Johnson et al. (2003) and Phillott et al. (2010). Survival of Bsal is likely to be minimal if water is disposed into the sewage system, as water treatment processes are likely to kill the zoospores.

This measure can also be applied to all effluent and waste materials in a manner that inactivates pathogenic fungi (Wombwell, 2014).