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Literature review on the main existing structures and systematic/academic initiatives for surveillance in the EU for zoonoses in the environment and the methods for surveillance of pathogens in the environment

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Abstract

A small proportion of disease surveillance programs target environment compartment, and in the EU these are restricted to few countries. The present report is composed of two literature reviews (i) on the main existing structures and systematic/academic initiatives for surveillance in the EU for zoonoses in the environment, and (ii) on the methods for pathogen surveillance in the environment. Concerning (i), it is noteworthy that the most frequently reported objective was to evaluate control and eradication strategies and following trends of zoonosis. However, detecting new pathogens or unusual epidemiological events were scarcely reported as objectives, as well as demonstrating freedom from a particular pathogen, despite the big potential that environmental sampling and testing techniques have recently demonstrated for these purposes. Few of the pathogens prioritised by EFSA were represented in this literature review, indicating the potential of environmental techniques to be applied to a larger extent to detect relevant transboundary and (re)emergent zoonoses. The preferred environmental sample was water, followed by biological material (included faecal material) and vectors (mosquitoes). To a much lesser extent, soil, and other matrices were used. Regarding (ii) the pathogen detection and identification methods were divided into: conventional (culture and biochemistry-based, and immunology-based); molecular methods (nucleic acid-based methods); biosensor-based (new) and others. A large percentage of available assays for the detection and surveillance of pathogens in the environment focuses on hazards that are not among those pre-selected by EFSA. Therefore, there is a need for development of new, untested, methods for surveillance of listed pathogens of higher epidemiological importance. Less disturbed areas, natural and wild environments are less covered by environmental sampling techniques than urban and farm environments and should therefore receive higher attention since they may hold undiscovered and potentially epidemiologically significant hazards and hosts. In general, molecular methods, namely the nucleic-acid based methods, are the ones more commonly and widely used for pathogen detection in environmental samples, and can be developed for virtually any organism, given a sufficient effort to identify specific DNA/RNA sequences unique to the target organism. The usefulness and appropriateness of different environmental matrices for detecting specific pathogens or for specific purposes are discussed and recommendations are provided.

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Key words: Literature review, zoonosis surveillance, non-invasive methods, environment

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Summary

Background: The EU-Commission is setting up a coordinated surveillance system under the One Health (OH) approach for cross-border pathogens that threaten the Union. To provide scientific and technical advice and improve future schemes of surveillance, this report present two literature reviews focused on environmental surveillance: (i) on the main existing structures and systematic, and academic initiatives for surveillance in the EU for zoonoses in the environment; and (ii) on the methods for surveillance of pathogens in the environment, respectively. A separate report includes a questionnaire on official surveillance on the main existing structures and systematic or academic initiatives activities for surveillance in the EU for zoonoses in domestic animals and wildlife, which also includes the environment; the results of the questionnaire are also contextualised in this report. It was shown that only a small proportion of surveillance programs (SPs) collect environmental samples (<10%) and this sampling is conducted by only some countries.

Methods:

- Literature review on the main existing structures and systematic/academic initiatives for surveillance in the EU for zoonoses in the environment.

  The purpose of this literature review was to collect published information regarding surveillance in Europe that focuses on health hazards where environmental sampling is involved. It includes a review of study design and methodological approaches, including types of environmental samples, and methods to detect animal pathogens in the environment. Documents describing systematic, structured, or academic surveillance systems targeting zoonotic pathogens in the environment in Member States and neighbouring countries were searched. A list of target zoonotic pathogens has already been produced by EFSA and was included in the search strings in literature browsers. The publication search was done in the following databases: Biomedical databases (Embase) and Science databases (ISI web of Science, Pubmed). In addition to references retrieved directly from scientific browsers the literature cited to identify missing references was examined. A standardised data model was used to extract key information to characterise the surveillance systems.

- Literature review on methods for surveillance of pathogens in the environment

  The purpose of this literature review was to assess the published information on the methods applied to survey zoonotic pathogens or diseases using environmental protocols, i.e., non-invasive environmental samples. To achieve this goal, scientific databases such as Scopus, Pubmed and Web of Science (WOS) were used. The search method was limited to documents published between 2017 to 2022. Thereafter, inclusion/exclusion criteria were applied. A standardised data model was similarly used to extract key information from each document: target pathogen; type of samples collected (faecal, object surface…) and if it is an invasive or non-invasive collection method; sample collection methods; pre-analytical treatments; pathogen identification methods, and existence of quality controls. Data were collected at the assay level. The full text of all selected papers was read, and relevant information was extracted, summarised, and schematically outlined in tables in the form of one or multiple single-entry assays per reference.

Results:

- Literature review on the main existing structures and systematic/academic initiatives for surveillance in the EU for zoonoses in the environment.

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63 scientific publications were selected for the review after applying the inclusion criteria. The most frequent source of funding was national (48%) followed by European funding (19%). In a large proportion of the publications the source of funding was not given (22%). The coordination of the surveillance systems was recorded as either integrated or standalone. In 51% of the publications, it was reported that the surveillance system was integrated while in 48% it was independent/standalone. Universities participated most frequently on the environmental surveillance (79.4%), followed by research institutes (60.3%), public health services (54%), and official laboratories (52.4%). To a lesser extent the following institutions or categories participated: Hospital/doctors (25.4%), environmental agencies (20.6%), local institutions (12.7%), citizen science (4.8%), agricultural sector (3.2%), private veterinarians (3.2%), hunting sector (1.6%) and wildlife management (1.6%). The majority of programmes were conducted at the national and subnational level, these programs are occasional and often short lasting. The largest difference in the surveillance between different territories was observed in the sampling effort, followed by time, spatial resolutions, sampling methods and pathogen. In 95.2% of the surveillance systems the objective was to evaluate control or eradication strategies, in 55.6% the objective was to determine trends monitoring to improve knowledge, and in 47.6% was to determine trends monitoring to support intervention design or evaluation. Most surveillance systems applied either passive surveillance (26.9%) or combined active and passive surveillance 27.1%. Only 18.4% of the SPs were exclusively based on active surveillance. Risk-based sampling was most often represented (55.6%), followed by random sampling (44.4%) and stratified sampling (34.9%). The type of environmental sample (matrix) used was recorded as: water, soil, air, biological material (including pellet/excrements), mosquitoes, environmental ticks, other vectors. Water was most often used (55.6%) followed by biological material (31.7%), mosquitoes (22.2%), soil (9.5%) and other samples to lesser extent.

- Literature review on methods for surveillance of pathogens in the environment

After a preliminary search using the defined strings 1253 publications were retrieved. However, after limiting the search with the inclusion and exclusion criteria 191 publications were included for the review. After reading and analysing these 191 publications a total of 686 assays were assessed for pathogen surveillance in the environment and relevant information was retrieved. A large percentage of available assays for the detection and surveillance of pathogens in the environment focuses on hazards that are not among those pre-selected by EFSA. However, these may be a source for development of new, untested, methods for the surveillance of those listed pathogens of higher epidemiological importance.

Type of environmental sample (matrix)

Almost half of the recorded assays for the detection of pathogens in the environment were applied and tested for surveillance in urban areas. This is in accordance with the higher epidemiological and health risks for human populations and the recent pandemic state, which caused a surge in epidemiological studies in populated areas. The same applies to explain the higher frequency of assays implemented for the surveillance of animal farm/market areas, given their connection to food and health safety, and the importance of biosecurity for animal production. Nevertheless, less disturbed areas, natural and wild environments may hold undiscovered and potentially epidemiologically significant hazards and hosts and should receive higher attention.

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In the studies of animal farm/market areas the object surface and water are the most common sample matrices. In urban areas, the wastewater has many possible contamination sources, giving a complex image of the environment/population. In natural areas, the non-invasive sampling of wildlife faeces can give information on possible hosts/new hosts, vectors, and emerging epidemics. Moreover, despite a very complex sample matrix, water can be the base of more holistic epidemiological studies, especially with the emergence of high-throughput molecular methods, such as NGS.

Collection methods

Most of the analysed assays were based in grab-sample method, i.e., simple collection of the environmental sample into a sterile container, this is the most simple and ubiquitous method. However, it requires higher sample quantity, large sample containers, people to collect the samples, higher storage capacity, among other requirements. The eDNA techniques have been extensively used in water samples allowing to detect multiple pathogens but also the animal hosts, namely vertebrate and invertebrate, and thus giving relevant information on the biodiversity. Thus, water is considered the most relevant sample matrix for pathogen surveillance in the environment. Sampling using swabs appears to be increasing, it requires lower quantity, less storage capacity and handling, and can be used in surfaces, and it can be embedded with substances for sample conservation. Nevertheless, it needs people to collect the samples and specific sterile material, which can be more expensive than simpler sterile containers as used in grab samples, as well as it is not possible to automatise. Despite not being widely used yet, air sampling can be done using effective automatic sampling, i.e., automatic pumps and filters/membranes, or newly developed variations. However, these may be costly and ineffective for the surveillance of pathogens of higher mass that fall onto surfaces. Nevertheless, air sampling can be used for detecting pathogens transmitted by air, namely viruses and some bacteria. The use of airborne methods is increasing due to their capacity of applying automatic and continuous sampling. Moreover, the automatic sampling may allow assessing larger areas in shorter time.

Pre-analytical treatment

In general, and independently of the sample matrix, pre-analytic treatments are used before pathogen detections. Pathogen isolation, whether it is in cells, eggs, or agar, helps to remove inhibitors for more complex molecular analysis, being most often employed for the characterization of samples for the detection of the pathogen. Performed mainly for viruses and bacteria, can also improve the assessment of the level of infection. Sample storage conditions are an important step in pathogen detection in the environment. Storage conditions are dependent on how long the samples will remain stored prior to analysis. The ideal scenario is the immediate analysis of the sample, especially for molecular methods. Nevertheless, when immediate analysis is not possible, samples are maintained cold and/or frozen at -20 or even at -80ºC if they need to be stored for longer periods prior analysis. This is mainly necessary when analysing viruses and bacteria. The freeze-thawing technique helps in the recovery of genetic material from oocysts/eggs from protozoa. Ultrafiltration has been recognized as an effective procedure for concentration and recovery of microbes from large volumes of water and treated wastewater. Method optimization, namely removing inhibitors, is very important to improve the detection success and its efficiency, especially in the DNA/RNA-based methods.

Pathogen detection, discrimination, and identification

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We have divided the pathogen detection and identification methods into: conventional (culture and biochemistry-based, and immunology-based); molecular methods (nucleic acid-based methods); biosensor-based (new) and others.

Conventional methods: Culture and Biochemistry-based methods tend to be more time consuming and sometimes inconclusive, with multiple stages of culture and testing being needed for confirmation. They provide less taxonomical information and are used as preliminary testing for other identification methods (molecular). Usually they are gold-standard. The immunology-based methods require less time to prepare the assay than a culturing technique. However, real-time pathogen detection is not possible with this method. Generally, immunoassays are performed before directly conducting polymerase chain reaction (PCR) based methods. HA is the more commonly used method in this case. Immunology-based methods may have general lower specificity, one factor that affects the specificity of the assay is the specificity of antibody. However, since polyclonal antibodies have polyvalency (multiple epitopes to react with), they can be used for a preliminary pathogen trial detection. Nevertheless, their use can affect the reaction, leading to low specificity and sensitivity. It must be noted that false positive results may occur. To overcome high detection limits, enrichment steps become important for the detection of pathogens in food products. In the enrichment step, a label-free immunoassay is used that helps in detecting the presence of the pathogen in a much simpler way. A simple and rapid detection is possible through this method with simultaneous enrichment and optical detection. The principle of this method is culture/capture/measure.

Molecular methods: molecular methods, namely nucleic-acid based methods are commonly and widely used for pathogen detection in environmental samples. There are several specific methods and they can be used as target or multiple pathogen detection. The nucleic-acid based methods can be developed for virtually any organism given a sufficient effort to identify specific sequences unique to the target organism. There are several conventional PCR techniques, which are simpler, not expensive, and well established. However, the real-time and/or quantitative PCR has been proven to be more time effective with very good specificity. Thus, these methods are by far the most used methods for detecting pathogens in non-invasive environmental samples. Moreover, the nucleic-acid-based techniques can provide accurate assessment of potential hosts, which can be used simultaneously when detecting target or multiple pathogens. Nevertheless, inhibitors in environmental samples can limit the nucleic-acid-based techniques, but there are methods for removing inhibitors. Moreover, the use of a positive control can help in the detection of false negatives and assess the role of possible inhibition in the reaction.

Biosensor-based methods: Biosensor-based methods have been developed, and they allow the accurate detection of pathogens, without the need of DNA/RNA extraction and amplification methods. As an example, the B1-LF-RPA, DNA-AuNP probe assay and the Radiometric colorimetric and AgNPs-fluorescence dual mode sensing for DPA based on Eu3+ have been used in pathogen detection. In recent years, great advances have been made in nanomaterial-based biosensors, where the sensing electrode is modified by a nanomaterial to achieve a quick electron transfer due to the stimulation of different biomarkers. Due to this advantage, research has been reported where the nanomaterials are coupled with biomolecules to develop nanomaterial-based biosensors to detect dangerous pathogens. *Salmonella*, *E. coli* and *L. monocytogenes* are the most studied pathogens; mainly food borne, where these methods have been used for their detection

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in food. However, respiratory syncytial virus and parasites (*Giardia* and *Cryptosporidium*) are other pathogens that have been also detected using biosensor-based methods. These recent modern techniques have increased sensitivity, selectivity, and stability, and allows a low detection limit for in situ measurement.

Pathogen characterization (epidemiology)

Pathogen isolation, and posterior genetic characterization have been shown to be crucial to understand pathogen evolution. Although the quantity and the quality of pathogens are usually lower in non-invasive environmental than in invasive samples, the recent development of detection, isolation and sequencing techniques allowed pathogen characterization. The high throughput sequencing techniques allow assessment of degraded DNA/RNA samples, and thus potentiate the use of nucleic-acid-based techniques associated with genome sequencing.

Discussion

- Literature review on the main existing structures and systematic/academic initiatives for surveillance in the EU for zoonoses in the environment.

  It is noteworthy that the most frequently reported objective was to evaluate control and eradication strategies and detect trends of zoonosis. However, detecting new pathogens or unusual epidemiological events was scarcely reported, likewise was demonstrating freedom from a particular pathogen, despite the big potential that environmental techniques have recently demonstrated for these purposes. Concerning the list of pathogens prioritised by EFSA, only few were represented in this literature review, which may indicate that the potential of environmental techniques still has to be developed and applied to detect these pathogens, which are considered relevant transboundary and emerging zoonoses. The preferred environmental sample was water, followed by biological material, which included faecal material, and vectors (mosquitoes). To a much lesser extent, soil, and other matrices were used.

- Literature review on methods for surveillance of pathogens in the environment.

  The pathogen detection and identification methods were divided into: conventional (culture and biochemistry-based, and immunology-based); molecular methods (nucleic acid-based methods); biosensor-based (new) and others. A large percentage of available assays for the detection and surveillance of pathogens in the environment focuses also on hazards not among those pre-selected for the prioritisation exercise by the OH working group of EFSA. However, these may pose an opportunity to develop new, untested, methods for the surveillance of those listed pathogens of higher epidemiological importance.

  Most of the pathogen surveillance assays in the environment were conducted in urban areas, followed by animal farms/markets. This indicates that emergent techniques to detect pathogens in the environmental are mainly applied in anthropized areas, where (i) environmental pathogen monitoring may be useful to detect increasing spread or fade out, and/or (ii) the risk of contact with environmental sources of zoonosis or pathogens that affect livestock are perceived as relevant or high, especially after the recent pandemic. These studies are essential to improve biosecurity in humans and animals. However, only 19.5% of the analysed assays were conducted in natural areas, which contrasts with the fact that most emergent zoonosis finds their main host reservoir in the wild. The most used sample matrix varied according to the environment (in animal farms/markets the object surface, in urban

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areas the wastewater, in the natural areas, the water) and the group of surveyed pathogens. Water-based samples were the most common matrix undergoing pre-analytical concentration. Nevertheless, the majority of the analysed assays have not used an inhibitor search or removal prior to pathogen analysis.

In general, molecular methods, namely the nucleic-acid based methods, are the ones more commonly and widely used for pathogen detection in environmental samples. There are several specific methods, which can be used for target or multiple pathogen detection. The nucleic acid-based methods can be developed for virtually any organism, given a sufficient effort to identify specific DNA/RNA sequences unique to the target organism. Among the nucleic-acid-based methods, real-time and/or quantitative PCR has been proven to be more effective and having a very good specificity. Thus, these methods are by far the most commonly used for detecting pathogens in non-invasive environmental samples. Moreover, nucleic-acid-based techniques can provide accurate assessment of potential hosts, which can be used simultaneously when detecting the target pathogen or multiple pathogens. This allows performing integrative pathogen surveillance in environmental samples, which needs to be better explored. Water analysis in farms and natural ponds, which may be used simultaneously by domestic and wild animals, is a good example where new research should be conducted. Case studies using environmental samples (water, soil, surface, and air) should be promoted, including the detection of multiple hosts using metagenomic approaches. Finally, the use of biosensor-based methods has increased in the last years, are very promising, and should be better explored, since they allow accurate detection of pathogens, without the need of DNA/RNA extraction and amplification methods.

The main **RECOMMENDATIONS** for further implementing surveillance of zoonotic pathogens in the environment are:

- The use of environmental techniques to detect pathogens in SPs has mainly been applied to evaluate control and eradication strategies and detecting trends of zoonosis. Proven their sensitivity to detect pathogens, we also recommend the use of these techniques to detect new pathogens or unusual epidemiological events.
- Only a short proportion of SPs collects environmental samples to detect pathogens and are restricted only to some countries; and the detection and surveillance of pathogens in the environment remain untested in most for the listed pathogens of higher epidemiological importance. We recommend incorporating environmental techniques to current SPs focusing on zoonotic prioritised pathogens since this has been done in only a minority of the SPs. Results must be compared with conventional surveillance in such ongoing SPs to evaluate their sensitivity and cost/effectiveness, especially for early warning/detection of zoonotic pathogens.
- The integration and use of environmental pathogen detection by different SPs and health sectors is recommended to address multi-pathogen multi-host disease surveillance. This approach can benefit from the combined use of different types of environmental samples to detect pathogens (such as water and biological materials, to a lesser extent soil, and other matrices), and should include vectors as matrices where pathogens can also be detected.
- The use of environmental techniques to detect pathogens is highly recommended for natural habitats where most host reservoirs of the listed priority zoonosis are present, targeting the appropriate sample matrix, such as water-based samples.

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- Nucleic acid-based methods can be developed for virtually any organism, given a sufficient effort to identify specific DNA/RNA sequences unique to the target organism, and can also provide accurate assessment of potential hosts, which can be used simultaneously when detecting the target or multiple pathogens. It is recommended that this approach is better explored and tested to perform integrative pathogen surveillance in environmental samples (e.g., the European Observatory of Wildlife).
- Water analysis in farms and natural ponds, which may be used simultaneously by domestic and wild animals, is a good example where new research should be conducted. Case studies using environmental samples (water, soil, surface, and air) should be promoted, including the detection of multiple hosts using metagenomic approaches.
- Finally, the use of biosensor-based methods has increased in the last years, they are very promising and should be better explored since they allow the accurate detection of pathogens, without the need of DNA/RNA extraction and amplification methods.

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1. General Introduction

1.1. Background and Terms of Reference as provided by the requestor

This contract was awarded by EFSA to Universidad de Castilla-La Mancha, contract title: Wildlife: collecting and sharing data on wildlife populations, transmitting animal disease agents, contract number: OC/EFSA/ALPHA/2016/01 – 01.

The terms of reference for the present report (specific contract 10, task 7. *Ad hoc* requests in systematic literature review, scientific and technical advice on targeted wildlife surveillance), are, as indicated in deliverable 2.2: "Describing and mapping of the main existing structures and systematic initiatives/academic activities for surveillance in the EU for zoonoses (transboundary, emerging and re-emerging) in domestic animals, wildlife, and the environment". The task should be based on review and a questionnaire survey on zoonotic disease (non-foodborne, non-AMR) surveillance activities in the EU, including any surveillance activity that focuses on zoonotic/emerging pathogens in animals (domestic animals, wildlife) as well as surveillance activities in the environment (environmental samples and vectors), even if only one sector is involved (domestic animals, wildlife, environment).

The deliverable consists of a scientific report with description of methods applied and the description of surveillance systematic initiatives and academic initiatives targeting transboundary zoonotic and emerging hazards in domestic animals, wildlife, and the environment. This deliverable is presented in two separate reports:

- **Report 1**: *Describing and mapping of the main existing structures and systematic initiatives and academic activities for surveillance in the EU for zoonoses (transboundary, emerging and re-emerging) in* ***domestic animals and wildlife***. A questionnaire on official surveillance was complemented by several literature reviews on the main existing structures and systematic or academic initiatives academic activities for surveillance in the EU for zoonoses in domestic animals and wildlife, which also include the environment (results on environment are also commented here, in report 2).
- **Report 2** (the present): *Literature review on the main existing structures and systematic/academic initiatives for surveillance in the EU for zoonoses in the environment and the methods for surveillance of pathogens in* ***the environment***. It presents two literature reviews (i) on the main existing structures and systematic, and academic initiatives for surveillance in the EU for zoonoses in the environment, and (ii) on the methods for surveillance of pathogens in the environment, respectively.

The region of operation of the activities is the EU (and neighbouring areas/countries, where relevant, e.g., the Balkans). Target of the questionnaire survey should be to collate information on all sectors (human health, domestic animals, wildlife, environment) from each surveyed country. As for the methods for surveillance of pathogens in the environment, the scope is worldwide.

1.2. Scope of the report

The *ENETWILD* consortium (www.enetwild.com) implemented an EFSA funded project whose main objective has been the harmonization and collection of information regarding the geographical distribution and abundance of wildlife and wildlife diseases throughout Europe.

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The EU-Commission has allocated specific resources for EU Member states (MS) for setting up a coordinated surveillance under the One Health approach for cross-border pathogens that threaten the Union. In this context, the tasks requested by EFSA to *ENETWILD* under specific contract 10 are to identify, describe and learn lessons from existing coordinated/collaborative disease surveillance.

A separate report, based on a questionnaire on official surveillance in the EU for zoonoses in domestic animals, wildlife, and the environment, evidenced that only a short proportion of surveillance programs (SPs) collect environmental samples (<10%) and are restricted to only some countries. This report presents two literature reviews (i) on the main existing structures and systematic/academic initiatives for surveillance in the EU for zoonoses in the environment, and (ii) on the methods for surveillance of pathogens in the environment, respectively.

2. The pathogens

We included a list of zoonotic diseases pre-selected for the prioritisation exercise by the One Health (OH) working group of EFSA (see Table 1). Therefore, the first review of the report refers to surveillance programs (SPs) including at least one of the listed zoonotic pathogens:

Table 1. List of 50 zoonotic pathogen species/genera pre-selected for the prioritisation exercise by the OH working group of EFSA.

Target pathogens	Caused disease
Bacillus anthracis	Anthrax
Brucella (*B. abortus, melitensis, suis*)	Brucellosis (*B. abortus, melitensis, suis*)
Chikungunya virus	Chikungunya fever
SARS-Coronavirus type 2	COVID-19
Crimean-Congo haemorrhagic fever virus	Crimean-Congo haemorrhagic fever
Cryptosporidium spp.	Cryptosporidiosis
Eastern equine encephalitis virus	Eastern equine encephalitis
Ebola virus disease virus	Ebola virus disease
Echinococcus spp. (*E. granulosus, E. multilocularis*)	Echinococcosis (*E. granulosus, E. multilocularis*)
Erysipelothrix rhusiopathiae	Erysipelothricosis
Giardia spp.	Giardiasis
Burkholderia mallei	Glanders
Hantavirus	Hantavirus infection
Rickettsia helvetica	Helvetica spotted fever
Hendra virus	Hendra virus infection
Hepatitis E virus	Hepatitis E
Influenza A virus (Avian)	Influenza, avian
Influenza A virus (Swine)	Influenza, swine
Japanese encephalitis virus	Japanese encephalitis
Lassa virus	Lassa fever
Leishmania spp.	Leishmaniosis
Leptospira spp.	Leptospirosis
Borrelia burgdorferi	Lyme borreliosis

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Lymphocytic choriomeningitis virus	Lymphocytic choriomeningitis
Marburg virus	Marburg virus disease
Rickettsia conorii	Mediterranean Spotted Fever
MERS-Coronavirus	MERS
Monkeypox virus	Monkeypox
Rickettsia typhi	Murine typhus
Nipah virus	Nipah virus infection
Omsk haemorrhagic fever virus	Omsk haemorragic fever
Yersinia pestis	Plague
Possawan virus	Possawan virus infection
Coxiella burnetii	Q-fever
Rabies virus	Rabies
Rift Valley fever virus	Rift Valley fever
SARS-Coronavirus type 1	SARS
Orientia tsutsugamush	Scrub typhus
Shuni virus	Shuni virus infection
Sindbis virus	Sindbis fever
St. Louis encephalitis virus	St. Louis encephalitis
Thogoto virus	Thogoto virus infection
Tick-borne encephalitis virus	Tick-borne encephalitis
Toxoplasma gondii	Toxoplasmosis
Francisella tularensis	Tularemia
Usutu virus	Usutu virus infection
Venezuelan equine encephalitis virus	Venezuelan equine encephalitis
Wesselsbron virus	Wesselsbron virus infection
West Nile virus	West Nile fever
Western equine encephalitis virus	Western equine encephalitis

Subsequently, we detail the list of pathogens and their main characteristics of relevance for the purposes of describing and mapping the official zoonosis surveillance frameworks in Europe in this report (table 2).

Table 2. Main characteristics of relevance for the purposes of describing and mapping the official zoonosis surveillance frameworks in Europe in this report.

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Pathogen	Type pathogen	Vector borne	Main (primary) reservoirs	Main vectors	Domestic cycle	Peri-domestic cycle	Sylvatic cycle	Per-dom. & dom.	Peri-dom. & sylvatic	Peri-dom. & domestic & sylvatic	Pathogen life cycle
Bacillus anthracis	Bacteria	No	Environment		No	Yes	Yes	No	Yes	No	Saprozoonosis
Borrelia burgdorferi	Bacteria	Yes	Rodents	Ticks (*Ixodes*)	No	No	Yes	No	No	No	Metazoonosis
Brucella *(B. abortus, melitensis, suis)*	Bacteria	No	Wild and domestic ungulates, hares		Yes	No	Yes	No	No	No	Direct
Burkholderia mallei	Bacteria	No	Domestic equids		Yes	No	No	No	No	No	Direct
Chikungunya virus	Virus	Yes	Human (wild primates)	*Aedes*	No	No	Yes	No	No	No	Metazoonosis
Coxiella burnetii	Bacteria	Yes	Mammals, birds	Ticks	Yes	No	Yes	No	No	No	Metazoonosis
Crimean-Congo haemorrhagic fever virus	Virus	Yes	Wild and domestic mammals	Ticks	Yes	No	Yes	No	No	No	Metazoonosis
Cryptosporidium spp.	Protozoa	No	Environment, vertebrates (cattle)		Yes	No	Yes	No	No	No	Direct
Eastern equine encephalitis virus	Virus	Yes	Horse, birds	Culex and *Culiseta* mosquitoe	Yes	No	Yes	No	No	No	Metazoonosis
Ebola virus disease virus	Virus	No	Fruit bats (primates and other wild mammals)		No	No	Yes	No	No	No	Direct
Echinococcus spp. *(E. granulosus, E. multilocularis)*	Helminth	No	Wild and domestic canids, ungulates, and rodents		Yes	No	Yes	No	No	No	Cyclozoonosis
Erysipelothrix rhusiopathiae	Bacteria	No	Environment, a wide variety of wild and domestic animals, birds, and fish		Yes	No	Yes	No	No	No	Direct

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Francisella tularensis	Bacteria	Yes	Rodents, Lagomorpha	Ticks	No	No	Yes	No	No	No	Metazoonosis
Giardia spp.	Protozoa	No	Environment, wild and domestic mammals, and birds		Yes	No	Yes	No	No	No	Direct
Hantavirus	Virus	No	Wild rodents		No	No	Yes	No	No	No	Direct
Hendra virus	Virus	No	Fruit bats		No	No	Yes	No	No	No	Direct
Hepatitis E virus	Virus	No	Human, wild and domestic suids		Yes	No	Yes	No	No	No	Direct
Influenza A virus (Avian)	Virus	No	Wild waterfowl, domestic poultry		Yes	No	Yes	No	No	No	Direct
Influenza A virus (Swine)	Virus	No	Wid and domestic suids		Yes	No	Yes	No	No	No	Direct
Japanese encephalitis virus	Virus	Yes	Vertebrate hosts, primarily pigs (wild boar, pigs) and wading birds	Mosquitoes (*Culex tritaeniorhynchus*)	No	No	Yes	No	No	No	Metazoonosis
Lassa virus	Virus	No	Rodents (multimammate rat *Mastomys natalensis*)		No	No	Yes	No	No	No	Direct
Leishmania spp.	Protozoa	Yes	*L. infantum*: Wild and domestics mammals: lagomorphs, carnivores (other such as hedgehogs)	Sand flies	No	No	Yes	No	No	No	Metazoonosis
Leptospira spp.	Bacteria	No	Rodents		No	Yes	Yes	No	Yes	No	Direct
Lymphocytic choriomeningitis virus	Virus	No	Wild and domestic rodents (*Mus musculus)*		No	No	Yes	No	No	No	Direct

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Marburg virus	Virus	No	Fruit bats (primates and other wild mammals)		No	No	Yes	No	No	No	Direct
MERS-Coronavirus	Virus	No	Dromedary camels		Yes	No	Yes	No	No	No	Direct
Monkeypox virus	Virus	No	Primates, rodents		No	No	Yes	No	No	No	Direct
Nipah virus	Virus	No	Fruit bats		No	No	Yes	No	No	No	Direct
Omsk haemorrhagic fever virus	Virus	Yes	Wild rodents	Ticks	No	No	Yes	No	No	No	Metazoonosis
Orientia tsutsugamushi	Bacteria	Yes	Wild rodents (mainly Rattus, also peri-urban)	Trombiculid mites	No	No	Yes	No	No	No	Metazoonosis
Possawan virus infection	Virus	Yes	Wild rodents (also shrews, medium size mammals)	Ticks (*Ixodes, Haemaphysalis* spp)	No	No	Yes	No	No	No	Metazoonosis
Rabies virus	Virus	No	Red Foxes, bats		No	No	Yes	No	No	No	Direct
Rickettsia conorii	Bacteria	Yes	Dogs (Lagomorpha)	*Rhipicephalus sanguineus*	No	Yes	Yes	No	Yes	No	Metazoonosis
Rickettsia helvetica	Bacteria	Yes	Natural vertebrate reservoir host remains to be determined	*Dermacentor reticulatus* and other ticks (*I. ricinus*)	No	No	Yes	No	No	No	Metazoonosis
Rickettsia typhi	Bacteria	Yes	Rodents: *Rattus*	Oriental rat flea (*Xenopsylla cheopis*)	No	Yes	Yes	No	Yes	No	Metazoonosis
Rift Valley fever virus	Bacteria	Yes	Domestic ruminants and camels (wildlife reservoirs such as rodents, wild ruminants	Mosquitoes (mainly *Aedes* and *Culex* spp.)	No	No	Yes	No	No	No	Metazoonosis

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			or bats may also contribute)								
SARS	Virus	No	Probably bats		No	No	Yes	No	No	No	Direct
SARS-Coronavirus type 1	Virus	No	Probably bats		No	No	Yes	No	No	No	Direct
SARS-Coronavirus type 2	Virus	No	Probably bats		No	No	Yes	No	No	No	Direct
Sindbis virus	Virus	Yes	Birds (Grouse and passerines)	*Culex* and *Culiseta* mosquitoe	No	No	Yes	No	No	No	Metazoonosis
St. Louis encephalitis virus	Virus	Yes	Birds (Passeriformes and Columbiformes)	Mosquitoes *Culex*	No	Yes	Yes	No	Yes	No	Metazoonosis
Tick-borne encephalitis virus	Virus	Yes	Rodents (also insectivores and carnivores)	*Ixodes* ticks	No	No	Yes	No	No	No	Metazoonosis
Toxoplasma gondii	Protozoa	No	Environment, wild and domestic Felidae, warm-blooded vertebrates		Yes	Yes	Yes	Yes	Yes	Yes	Cyclozoonosis
Usutu virus	Virus	Yes	Wild birds	Mosquitoes *Culex*	No	No	Yes	No	No	No	Metazoonosis
Venezuelan equine encephalitis virus	Virus	Yes	Wild rodents (equines)	Mosquitoes *Culex*	No	No	Yes	No	No	No	Metazoonosis
West Nile virus	Virus	Yes	Wild birds	Mosquitoes *Culex*	Yes	Yes	Yes	Yes	Yes	Yes	Metazoonosis
Western equine encephalitis virus	Virus	Yes	Horse, birds	*Culex* and *Culiseta* mosquitoe	Yes	No	Yes	No	No	No	Metazoonosis
Yersinia pestis	Bacteria	Yes	Wild rodents	Fleas	No	No	Yes	No	No	No	Metazoonosis

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3. Literature review on the main existing structures and systematic initiatives for surveillance in the EU for zoonoses in the ENVIRONMENT

1.1. Methods

The purpose of this literature review was to collect published information regarding surveillance in Europe that focus on health hazards where environmental sampling is involved. It includes a review of study design and methodological approaches, including types of environmental samples, and methods to detect animal pathogens in the environment.

We aimed to search for documents describing systematic, structured, or academic surveillance systems targeting zoonotic pathogens in the environment in Member states and neighbouring countries. A list of target zoonotic pathogens has already been produced by EFSA, and was included in the search strings, as the following:

((Bacillus anthracis) OR Brucella OR Chikungunya OR (Crimean-Congo haemorrhagic fever) OR Cryptosporidium OR (Eastern equine encephalitis) OR (Ebola virus disease) OR Echinococcus OR (Erysipelothrix rhusiopathiae) OR Giardia OR (Burkholderia mallei) OR Hantavirus OR (Rickettsia Helvetica) OR (Hepatitis E) OR (avian influenza) OR (swine influenza) OR (Japanese encephalitis) OR Lassa OR Leishmania OR Leptospira OR (Borrelia burgdorferi) OR (Lymphocytic choriomeningitis) OR Marburg OR (Rickettsia conorii) OR MERS-Coronavirus OR Monkeypox OR (Rickettsia typhi) OR Nipah OR (Yersinia pestis) OR (Coxiella burnetiid) OR Rabies OR (Rift Valley fever) OR (Tick-borne encephalitis) OR (Toxoplasma gondii) OR (Francisella tularensis) OR Usutu OR (West Nile))

The publication search was done in the following databases:

- Biomedical databases (Embase)
- Science databases (ISI web of Science, Pubmed)

The details of search terms, and the use word string is presented in table 3.

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Table 3. Details in the search strings used for the indexed literature review.

Literature review	Concept to address	Target	Terms	String
Systems that regularly collect environmental samples in EU MS to detect environmental hazards	Environmental sampling	Title	"environmental sampling" OR environm*	Environmental sampling [Title] AND Hazards [Topic] AND Surveillance system [Title] AND Geography [Topic]
	Hazards	Topic (Title, Abstract, Keywords)	zoono* or disease or transbound or emerg*	
	Surveillance system	Title	surveillance OR monitor*	
	Geography	Topic (Title, Abstract, Keywords)	Albania OR Latvia OR Andorra OR Liechtenstein OR Armenia OR Lithuania OR Austria OR Luxembourg OR Azerbaijan OR Malta OR Belarus OR Moldova OR Belgium OR Monaco OR "Bosnia and Herzegovina" OR Montenegro OR Bulgaria OR Netherlands OR Croatia OR Norway OR Cyprus OR Poland OR "Czech Republic" OR Portugal OR Denmark OR Romania OR Estonia OR Russia OR Finland OR "San Marino" OR Macedonia OR Serbia OR France OR Slovakia OR Georgia OR Slovenia OR Germany OR Spain OR Greece OR Sweden OR Hungary OR Iceland OR Switzerland OR Ireland OR Turkey OR Italy OR Ukraine OR Kosovo OR "United Kingdom" OR Algeria OR Egypt OR Libya OR Morocco OR Sudan OR Tunisia OR Sahara OR Bahrain OR Cyprus OR Egypt OR Iran OR Iraq OR Israel OR Jordan OR Kuwait OR Lebanon OR Oman OR Palestine OR Qatar OR "Saudi Arabia" OR Syria* OR "United Arab Emirates" OR Yemen OR Europe OR European Union OR EU	

In addition to references retrieved directly from scientific browsers, we examined the literature cited to identify missing references. We finally obtained a total of 267 publications after the removal of duplicates (table 4).

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Table 4. Number of publications in different scientific databases focusing on zoonosis surveillance (transboundary, emerging and re-emerging in the environment across EU. The inclusion criteria are also presented (se also Figure 1a).

Outputs in ISI	Outputs in PubMed	Outputs in Embase	Total with duplicates removed	INclusion Criteria
140	168	78	267	1. Is the environmental sampling described in the paper? 2. Is the sampling applied in an EU MS or neighbouring country?

The data model

A standardized data model (see Annex 1[2]) was used to extract key information to characterize the surveillance systems. Variables were categorized, for which an associated vocabulary with definitions was developed (references sheet). The data model consists of a single sheet and the references and vocabulary are given in a separate sheet in the excel file that collected the information. This includes the list of countries and the list of diseases and respective pathogens involved in the literature review.

1.2. Results

1.2.1. General

Initially, 267 references were retrieved after removing duplicates. 63 scientific publications were selected for the review after applying the Inclusion criteria (Figure 1a).

Regarding the origin of funding, the proportion and number of SPs are summarized in Figure 1b The most frequent source of funding was national (48%) followed by European funding (19%). In a large proportion of the publications the source of funding was not given (22%).

[2] https://doi.org/10.5281/zenodo.7409275

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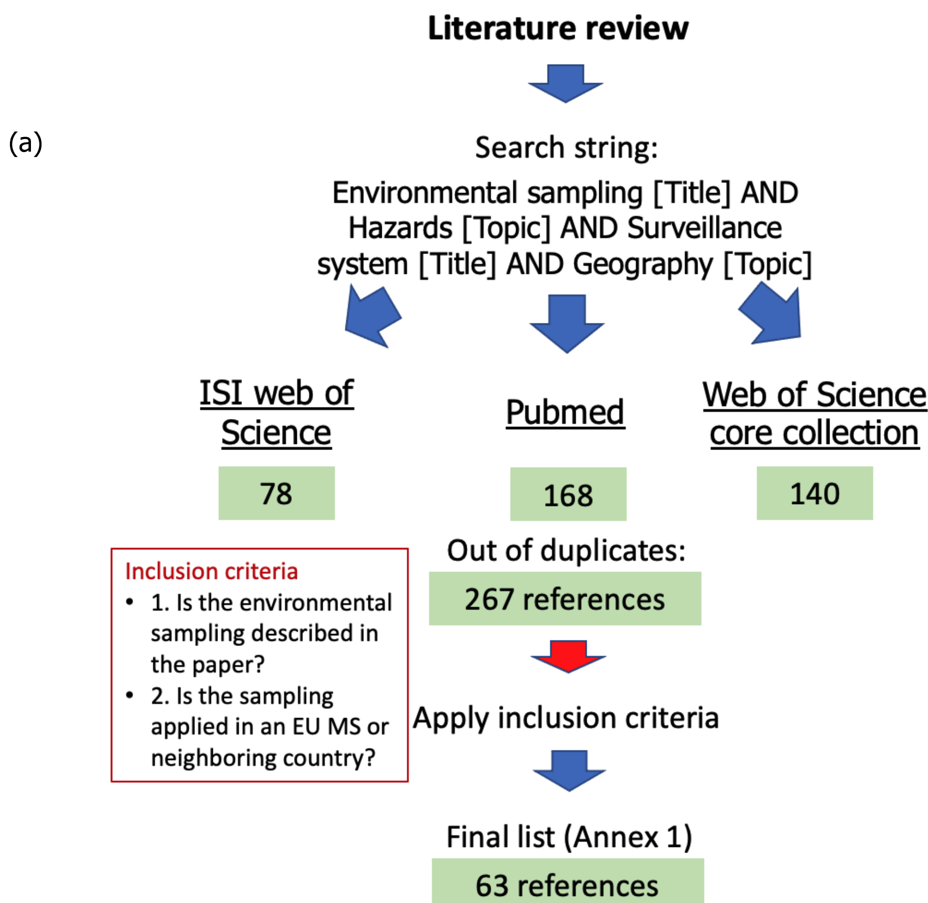

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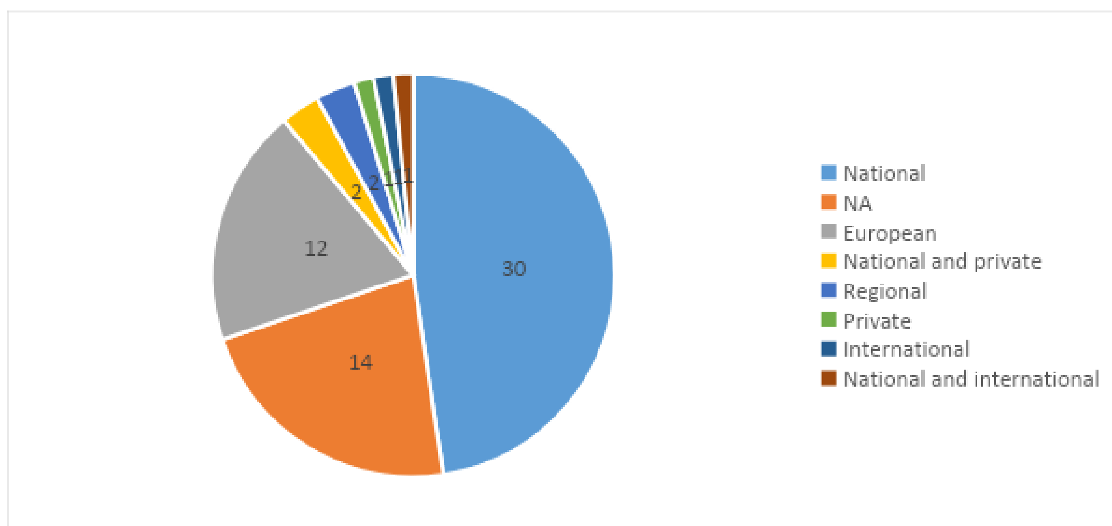

Figure 1. (a) Procedure and steps performed to review the literature on the main existing structures and systematic/ initiatives academic activities for surveillance in the EU for zoonoses in the environment. (b) The origin of funding (the proportion and number) of SPs (n=63).

1.2.2. Coordination

(b) rdination of the surveillance systems was recorded as either integrated or standalone. By i ed it meant that environmental surveillance was part of a combined surveillance system that involved other domains, i.e humans, domestic animals, wildlife. By standalone it is meant that the system was only for environmental surveillance and independent from other domains. In 51% of the publications it was reported that the surveillance system was integrated while in 48% it was independent/standalone (Figure 2).

The number of studies of either integrated or independent surveillance in relation to the type of funding is shown in Figure 2. While more standalone systems were financed nationally, integrated systems were financed by both national and European funding.

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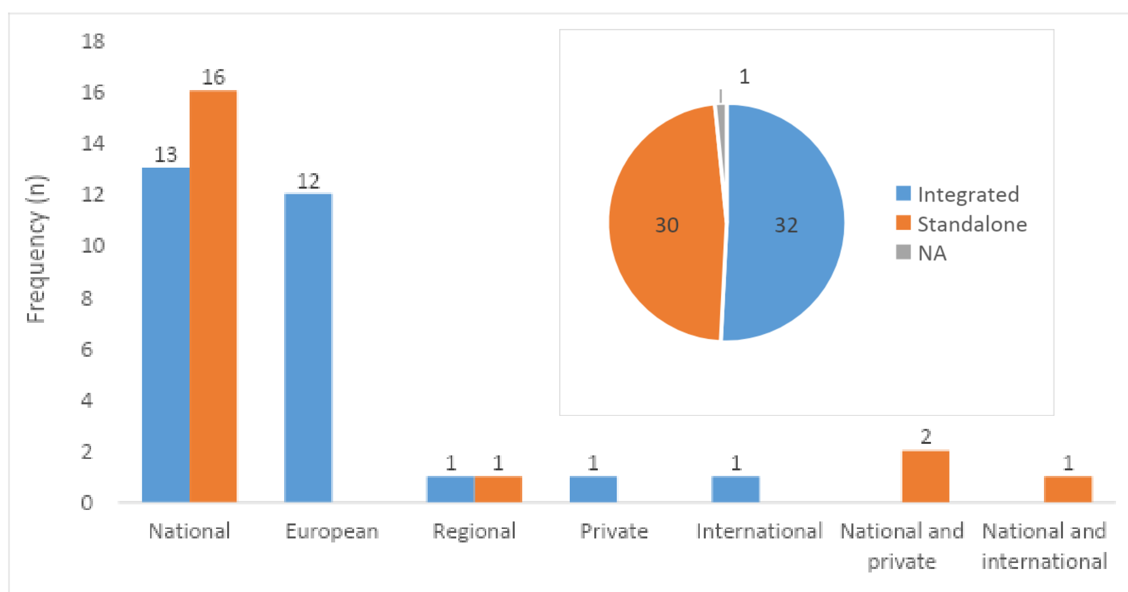

Figure 2. Variation of surveillance systems by their coordination status (integrated or standalone) and by the origin of funding.

1.2.3. Participating Institutions

The types of institutions that participated in the surveillances and the frequency of their participation were analysed (Figure 3). Universities participated most frequently in environmental surveillance (79.4%), followed by research institutes (60.3%), public health services (54%), and official laboratories (52.4%). To a lesser extent the following institutions or categories participated: Hospital/doctors (25.4%), environmental agencies (20.6%), local institutions (12.7%), citizen science (4.8%), agricultural sector (3.2%), private veterinarians (3.2%), hunting sector (1.6%) and wildlife management (1.6%). There were no reports of participation of pharmaceutical companies and/or wildlife rescue centres.

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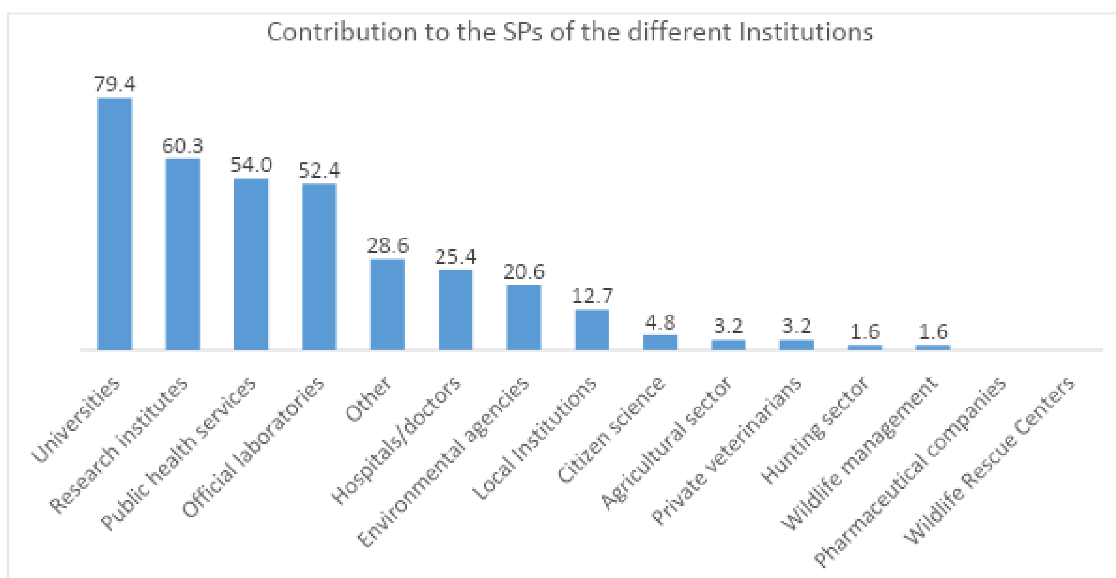

Figure 3. Diversity of institutions and their contribution or participation (frequency) in the pathogen environmental surveillance (N=63).

Several other institutions have participated (28.6%) on the SPs, namely:

- Waste water treatment facilities (n=11)
- Others:
  - Hellenic Centre for Disease Control & Prevention (HCDCP) (n=1)
  - Centers for Disease Control and Prevention, Atlanta, GA, USA (n=1)
  - Israel Min of Health (n=1)
  - Nursing homes for the mentally disabled (n=1)
  - Southeastern Italy's Regional Center for Epidemiology (OER) (n=1)
  - The Health Service of Italian Railways (n=1)
  - World Health Organization Eastern Mediterranean Regional Office, Amman, Jordan (n=1)

Nevertheless, it should be noticed that more than one institution was often involved in the pathogen environmental surveillance. The number of institutions participating in the SP mostly ranged between 3 and 4 (Figure 4).

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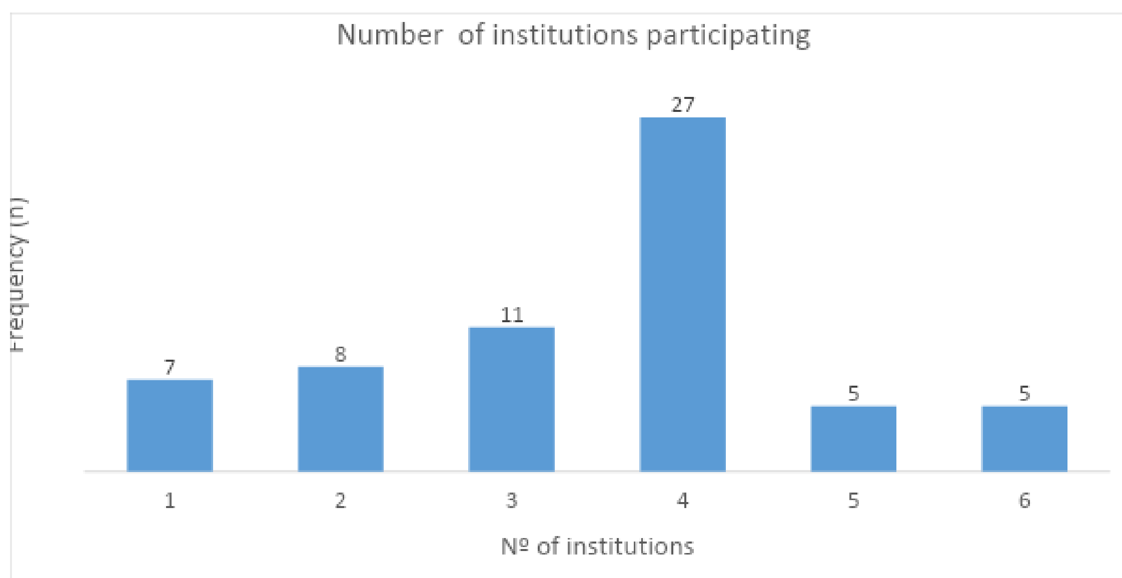

Figure 4. Number of institutions participating in the SP.

1.2.4. Geographical and temporal coverage

The level of geographical coverage of the surveillance was recorded as supranational, national or subnational. Most frequently the coverage was subnational (44%), followed by national (37%) and supranational (19%) (Figure 5).

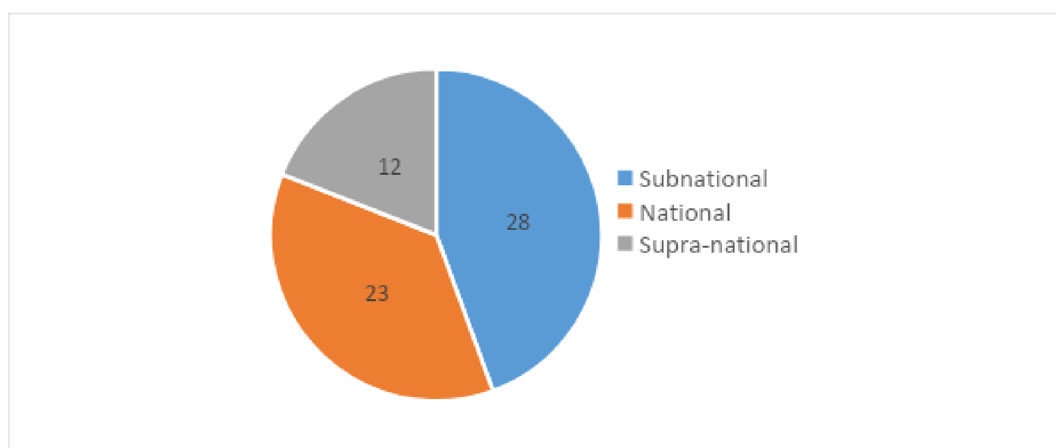

Figure 5. Number of surveillance systems per geographical coverage: supra-national; national and subnational (n=63).

The year of establishment of the first surveillance efforts was recorded and is shown in Figure 6. The publications described surveillance conducted between 1956 and 2018, but many were initiated in 2014.

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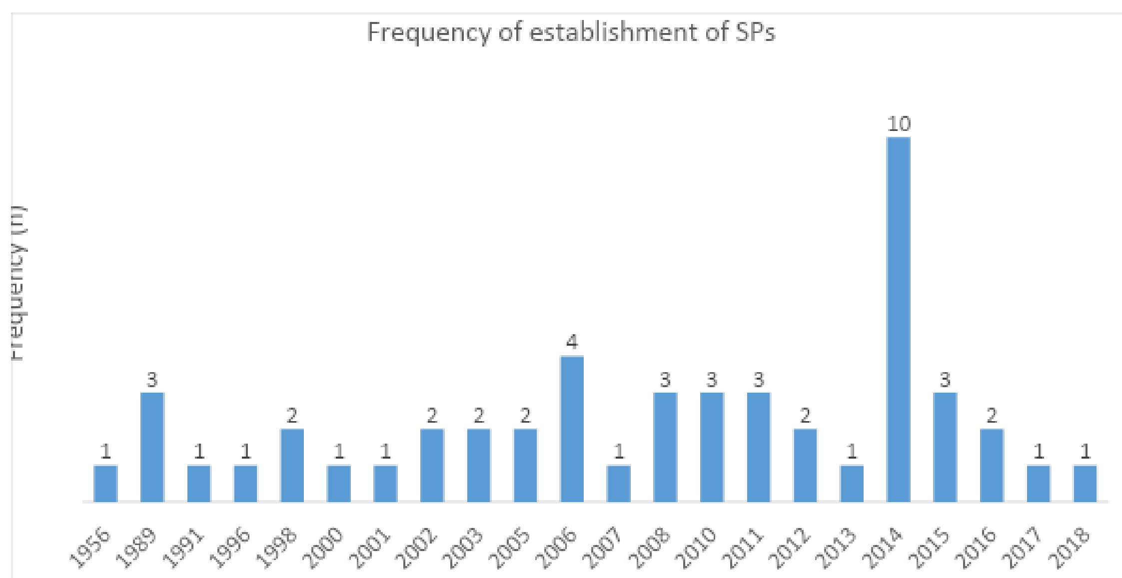

Figure 6. Timeline indicating the frequency of establishment of SPs (number by year).

The status of the described programs on environmental pathogen surveillance was reported as if ongoing or concluded. The majority of programmes at the national and subnational level were concluded, while most of the programmes at the supranational level were still ongoing at the time of publication (Figure 7).

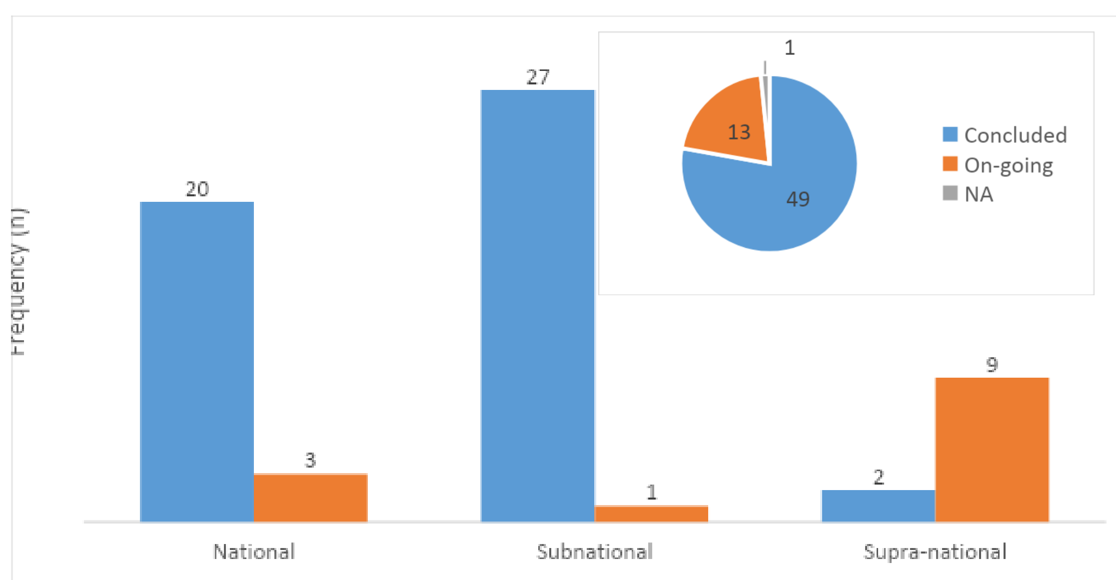

Figure 7. Number of environmental pathogen surveillance programs as concluded or on-going. Represented also in function geographical coverage.

It was recorded if programmes in different territories differ in any of the following aspects: sampling effort, time, spatial resolution, sampling methods, pathogen, target species and other factors. These differences, here termed "dis-homogeneities" are graphically presented in Figure

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8. The largest difference in the surveillance beween different territories was observed in the sampling effort, followed by time, spatial resolutions, sampling methods and pathogen. Target species differed less.

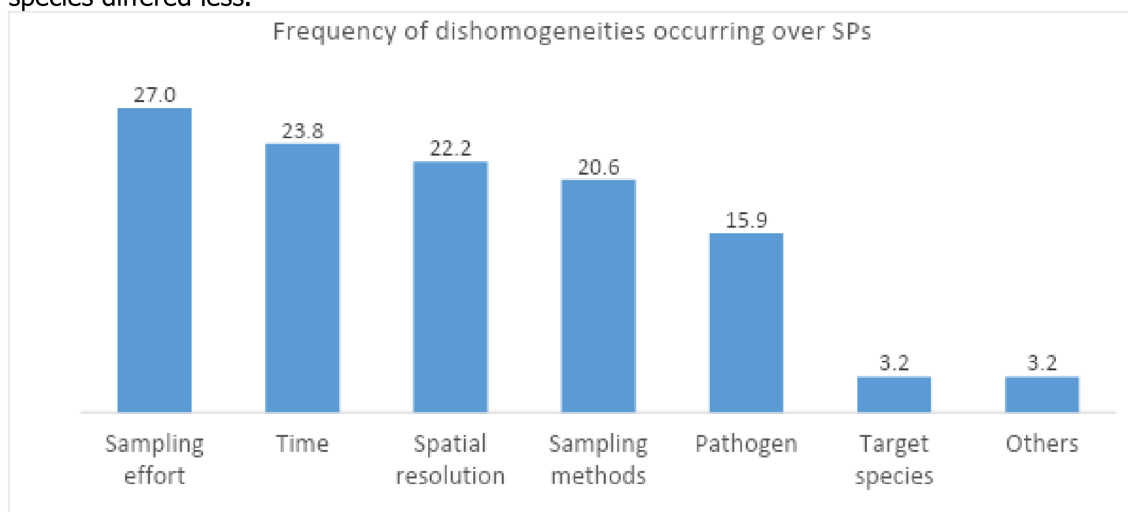

Figure 8. Frequency of dishomogeneities occurring at temporal and spatial resolutions over SPss (N=63). Others: Different categories of sectors/hospitals (time built and bed capacity)

1.2.5. Objectives

The objective(s) of the surveillance systems was recorded by selecting among the following categories: Early detection for rapid response, Trends monitoring for eradication or control, Trends monitoring to improve knowledge, Trends monitoring to support intervention design/evaluation and Monitoring of compliance with threshold values. More than one objective could be recorded. The frequency of the objectives given is presented below (Figure 9). In 95.2% of the surveillance systems the objective was to evaluate control or eradication strategies, in 55.6% the objective was Trends monitoring to improve knowledge and in 47.6% was Trends monitoring to support intervention design or evaluation.

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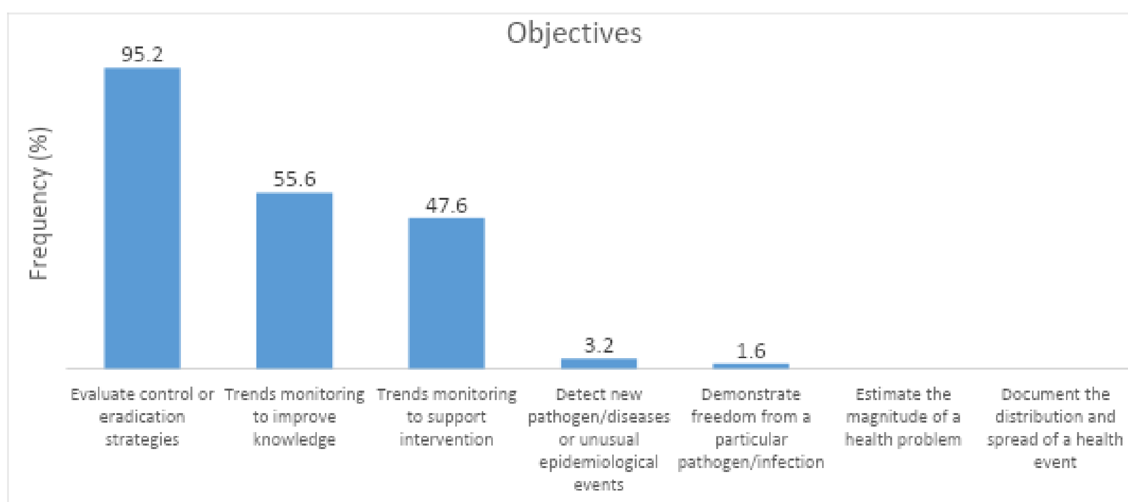

Figure 9. Frequency of different objectives (non-mutually exclusive) of the surveillance systems (N=63).

1.2.6. Evaluation of the surveillance systems

It was recorded if the surveillance system was evaluated or not. No evaluation was shown in 65% of the systems while 16% reported external evaluation and 14% internal evaluation (Figure 10).

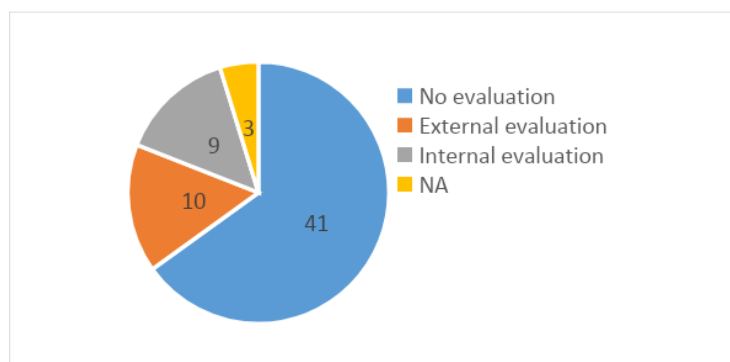

Figure 10. Existence of an evaluation process for the surveillance system and frequency (n=63)

Information was collected regarding the application of international standards in the SP, if the results of the evaluation were used to update/improve the system itself and if feedback was provided to stakeholders that are involved in direct sampling/disease detection. The frequency of these aspects evaluated in the surveillance system is shown below (Figure 11). International standards were applied in 61.9% of the systems, the results of the evaluation were used in 25.4% and reports to stakeholders were made in 23.8% of the systems.

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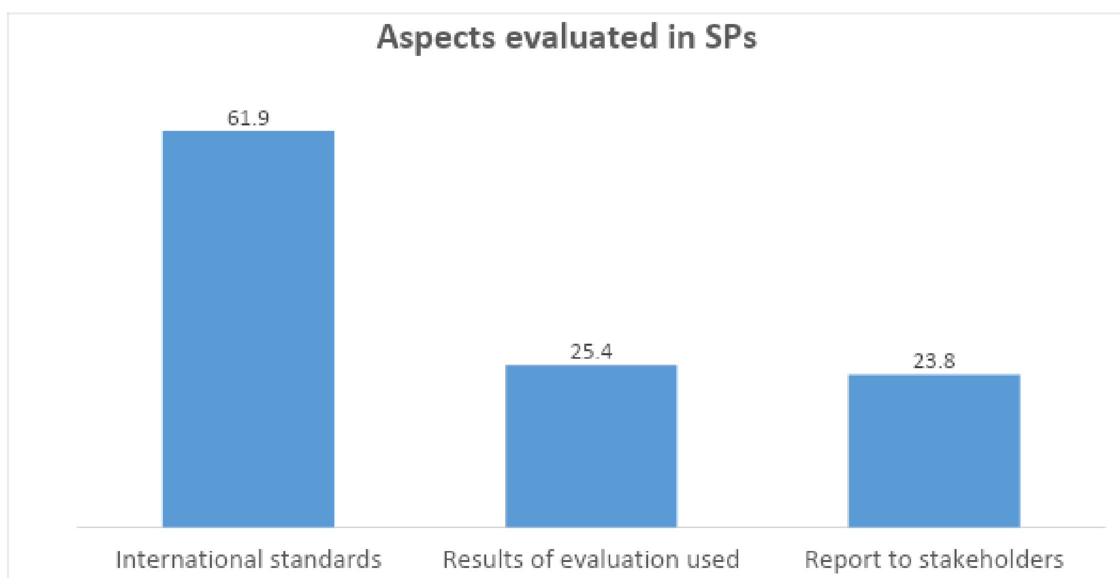

Figure 11. Aspects evaluated in SPs.

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1.2.7. Characteristics of surveillance

1.2.7.1. Active *vs* passive surveillance

Figure 12 displays the frequency of passive and active surveillance (or combined) applied by surveillance systems. Most surveillance systems applied either passive surveillance (26.9%) or combined active and passive surveillance 27.1%. Only 18.4% of the SPs were exclusively based on active surveillance.

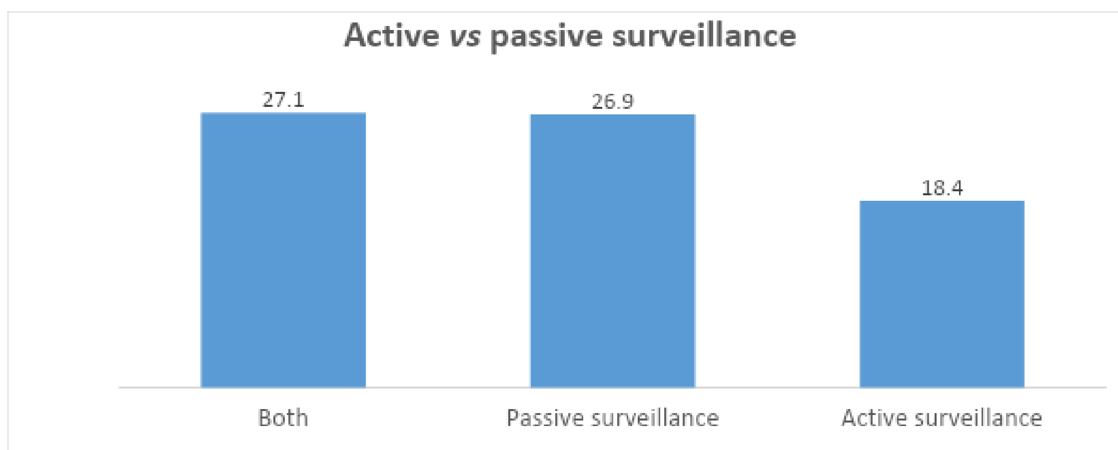

Figure 12. Frequency (%) of passive and active surveillance (or combined) applied by SPs.

1.2.7.2. Target hazards

The target hazards investigated by the surveillance were given and the list is shown below (Table 5, List of hazards and frequency, n=62)

Table 5. The target hazards investigated by the surveillance.

Pathogens on the list	Nº
Francisella tularensis	3
Usutu virus	1
West Nile virus	2
Chikungunya	1
Coxiella burnetii	1
Hepatitis E	1
Rift Valley fever	1
Sindbis virus	1
Pathogens not on the list	**Nº**
Legionella	11
Enterovirus	10
Toxocara	4
Non-polio enteroviruses	3

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Dengue	1
Yellow fever	1
Ancylostoma caninum	1
Aspergillus terreus	1
Candida parapsilosis	1
Chlamydophila psittaci	1
Hepatitis A virus	1
Nontuberculous mycobacteria (NTM)	1
Parechovirus	1
Reovirus	1
Saffold virus	1
Salmonella spp	1
Tahyna orthobunyavirus	1
Zika	1

Other hazards targeted by the surveillance are presented in Table 6.

Table 6. Other hazards targeted by the surveillance.

Other hazards	Nº
Invasive mosquito species	2
MRSA	1
Environmental quality	1
Hazardous chemicals	1
Micropollutants	1
Mortality, deprivation, cancer	1
Ixodes ricinus ticks	1
Streptomycin	1

The frequency of the type of hazard classified as virus, bacteria, helminths, fungus and other types targeted by the surveillance system was recorded and is shown below (Figure 13). Viruses were most often the target of the surveillance (44%) followed by bacteria (29%).

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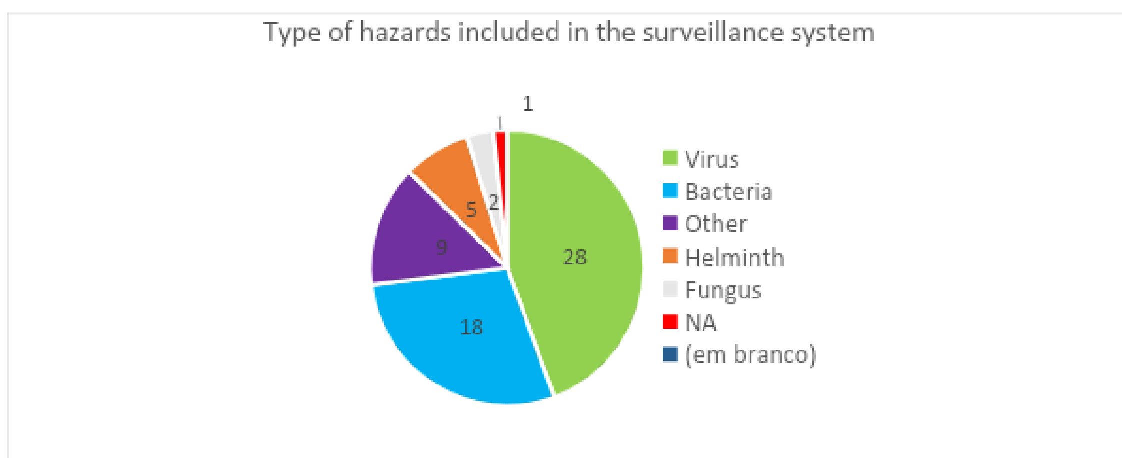

Figure 13. Frequency (n) of type of hazards included in the surveillance system (top).

1.2.7.3. Sampling design

The sampling design was recorded and is presented below (Figure 14). A surveillance system could use several sampling strategies simultaneously. Risk-based sampling was most often represented (55.6%), followed by random sampling (44.4%) and stratified sampling (34.9%).

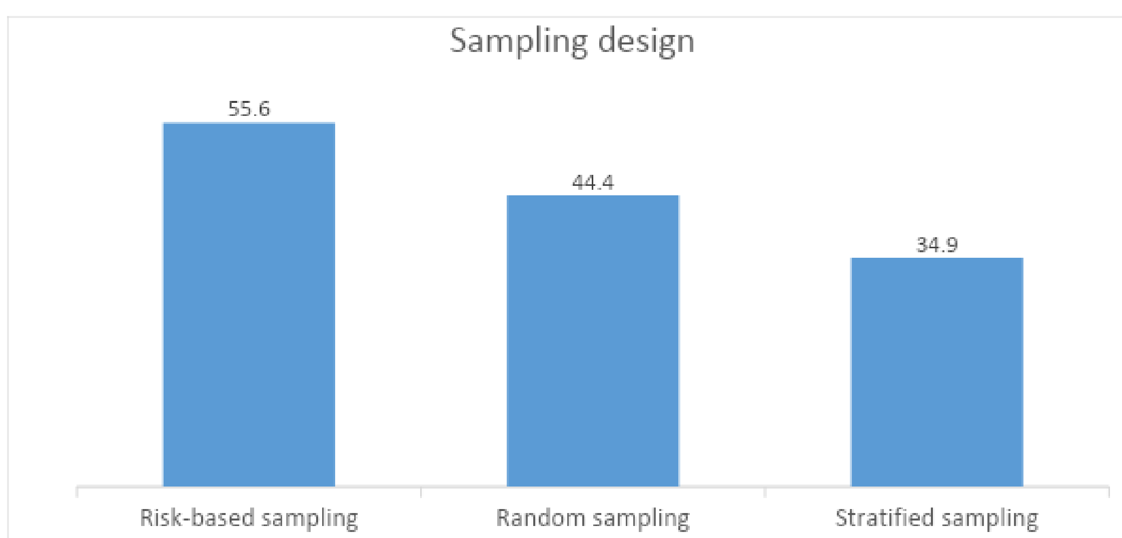

Figure 14. Frequency (%) of sampling design (non-mutually exclusive) of SPs.

1.2.7.4. Type of environmental sample (matrix)

The type of environmental sample (matrix) used was recorded as: water, soil, air, biological material (including pellet/excrements), mosquitoes, environmental ticks, other vectors. Water was most used (55.6%) followed by biological material (31.7%), mosquitoes (22.2%), soil (9.5%) and other samples to a lesser extent (Figure 15).

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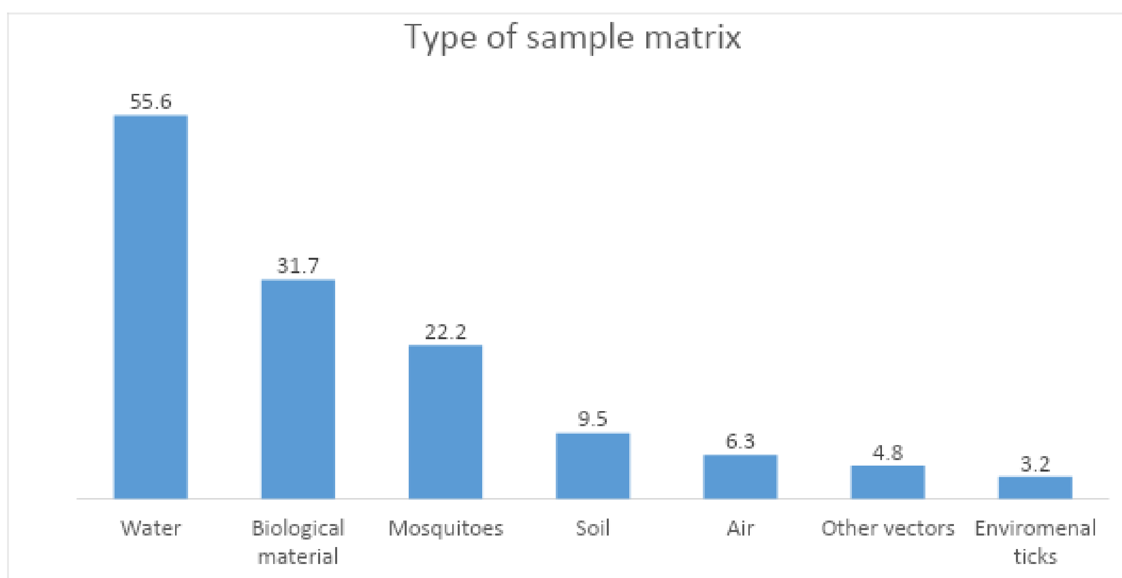

Figure 15. Frequency (%) of sampled matrix analysed in the surveillance systems.

1.3. Discussion

To remark the main differences against official surveillance, we evidenced that (i) the international component of funding is more relevant, (ii) that 50% of activities standalone for the environmental surveillance (and therefore would be not detected if we only focus on official surveillance, and (ii) about participating institutions, there was a predominance of Research institutes, followed by public health services and official laboratories. Also (iv) subnational geographical scale is more relevant, probably involving regions, big municipalities, and private companies (e.g., water). Interestingly, although no reason was evident, many programmes initiated in 2014. Like official surveillance, sampling effort is the main source of dishomogeneities among SPs/activities, also built methods, and where and when sampling is carried out. A relevant specificity (v) detected is that the frequency and ranking of different objective varies respect to official surveillance. It is noteworthy that the most frequently reported objective was to evaluate control and eradication strategies and detecting trends. However, detecting new pathogens or unusual epidemiological events was scarcely reported (and demonstrate freedom from a particular pathogen), in spite of the big potential that environmental techniques has recently demonstrated for these purposes (see review on methods).

As for official surveillance, active surveillance predominated, however (vi) it is remarkable that, about the selected pathogens, those included in the list of EFSA were minority in this literature review list. This may indicate that the potential of environmental techniques still has to be applied to detect these pathogens, which are considered relevant transboundary and emerging zoonoses. Similarly, risk-based design (57%) was most commonly used, followed by random and stratified. The preferred environmental sample was water, followed by biological material (included faecal material) and vectors (mosquitoes). To a much less extent, soil, and other matrices. The appropriateness of different environmental matrices for detecting specific pathogens or for specific purposes are discussed in the literature review on environmental methods.

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2. Literature review on methods for surveillance of pathogens in the environment

The purpose of this literature review was to assess the published information on the methods applied to survey zoonotic pathogens or diseases using environmental protocols. It includes a literature review on the different methods applied according to target animal pathogens using non-invasive environmental samples.

2.1. Methods

Our approach aimed at searching for published documents which describe methods for surveying pathogens in the environment, using non-invasive samples. The search was limited to the target zoonotic pathogens or diseases already produced by EFSA (see Table 7).

We searched for academic, peer-reviewed articles/documents, describing and/or using methods to detect animal pathogens in the environment. To achieve this goal, scientific databases such as Scopus, Pubmed and Web of Science (WOS) were used. In the search method, we limited our search in documents published between 2017 to 2022, using the following search string:

"Environmental sampling" AND "Hazards" AND "Surveillance system"

The search terms and strings used in this literature review are presented in **Table 7.**

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Table 7. Detailed search terms and strings used for the literature review on surveillance of pathogens in the environment.

Literature review	Concept to address	Target	Terms	String
Methods for surveillance of pathogens in the environment	Environmental sampling	Topic (Title, Abstract, Keywords)	("environmental sampl*") AND (protocol OR technique OR method)	Environmental sampling [Topic] AND Hazards [Topic] AND Surveillance system [Topic]
	Hazards (pathogens)	Topic (Title, Abstract, Keywords)	((Bacillus anthracis) OR Brucella OR Chikungunya OR (Crimean-Congo haemorrhagic fever) OR Cryptosporidium OR (Eastern equine encephalitis) OR (Ebola virus disease) OR Echinococcus OR (Erysipelothrix rhusiopathiae) OR Giardia OR (Burkholderia mallei) OR Hantavirus OR (Rickettsia Helvetica) OR (Hepatitis E) OR (avian influenza) OR (swine influenza)) NOT (residue* OR pesticid* OR contaminant* OR toxin*) ((Japanese encephalitis) OR Lassa OR Leishmania OR Leptospira OR (Borrelia burgdorferi) OR (Lymphocytic choriomeningitis) OR Marburg OR (Rickettsia conorii) OR MERS-Coronavirus OR Monkeypox OR (Rickettsia typhi) OR Nipah OR (Yersinia pestis) OR (Coxiella burnetii) OR Rabies OR (Rift Valley fever) OR (Tick-borne encephalitis) OR (Toxoplasma gondii) OR (Francisella tularensis) OR Usutu OR (West Nile)) NOT (residue* OR pesticid* OR contaminant* OR toxin*)	
	Surveillance system	Topic (Title, Abstract, Keywords)	(surveillance OR monitor* OR "surveillance program" OR "monitoring program")	

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As an initial dataset, we obtained a total of 1253 references after removing the duplicate references from the different databases. In order to limit the database, the described below inclusion/exclusion criteria were applied.

Inclusion/Exclusion criteria

The criteria used for including a document in the review were applied systematically and correspond to the following:

1. Refer to a pathogen in the EFSA list;
2. Use environmental samples to identify the pathogens;
3. Describe the laboratory method used to identify the pathogens.

The exclusion criteria applied to restring the obtained references were:

1. The document is a review

2. The document is in a language other than English

The procedure and steps performed to accomplish a final list of references to be analysed are summarized in Figure 16.

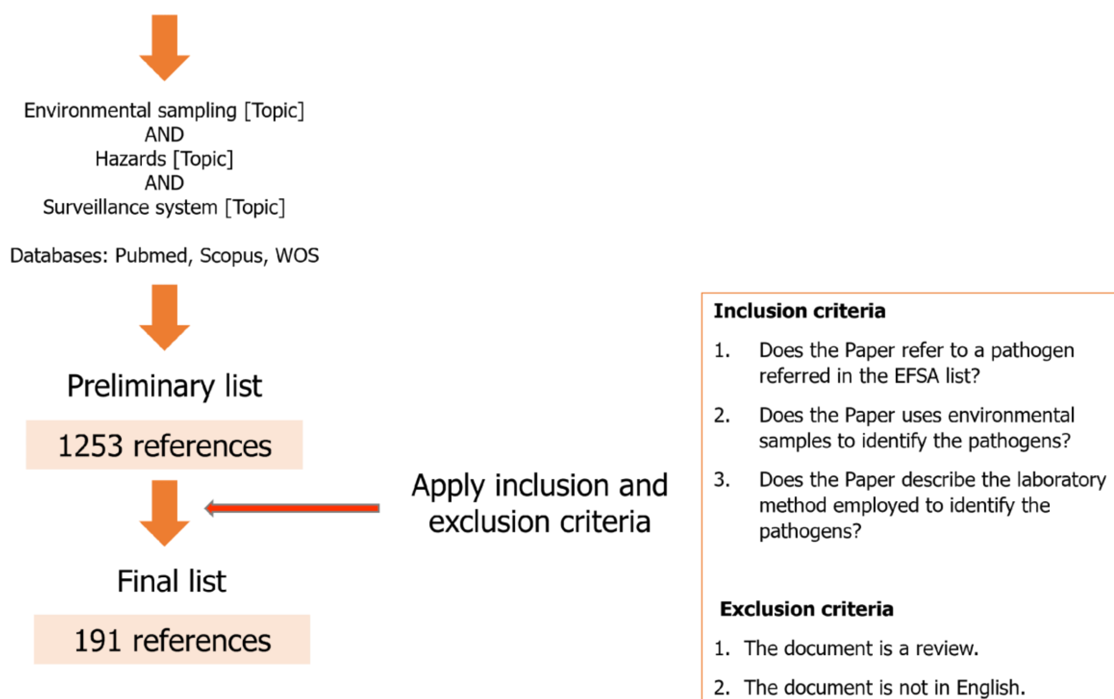

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Figure 16. Steps performed for the systematic literature review on the methods to detect animal pathogens in environment samples.

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The data model

A standardized data model, presented in Annex 2[3], was used to extract key information from each document, and it was divided into 6 parts:

- PART 1 – Target pathogen
- PART 2 – Type of samples collected (faecal, object surface…) and whether it is an invasive or non-invasive collection method
- PART 3 – Sample collection methods
- PART 4 – Pre-analytical treatments
- PART 5 – Pathogen identification methods
- PART 6 – Quality controls

2.1.1. Data analysis

Data was collected at the assay level. The full text of all selected papers was read, and relevant information was extracted, summarized, and schematically outlined in tables in the form of one or multiple single-entry assays per reference as schematized below (Figure 17).

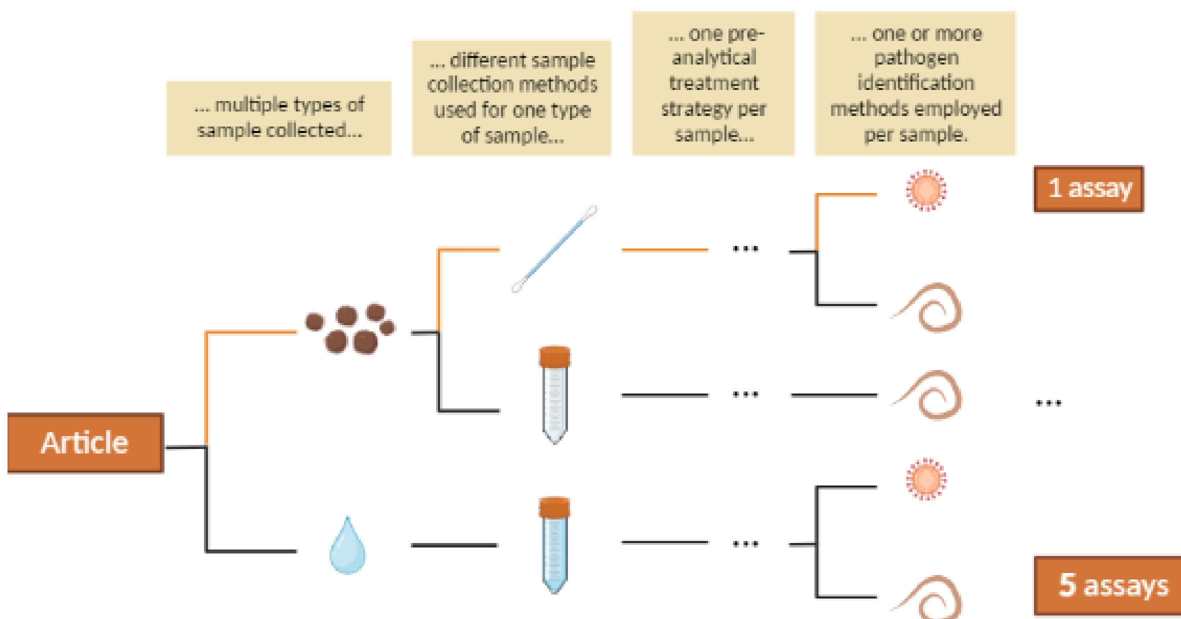

Figure 17. Example of the adopted steps in data collection and systematization.

[3] https://doi.org/10.5281/zenodo.7409275

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2.2. Results

2.2.1. General

Initially, 1253 references were obtained after the removal of duplicates. A total of 191 publications were selected for the review after the application of the referred inclusion/exclusion criteria (Annex 2[4]). The analysis of the 191 papers allowed assessing a total of 686 assays for pathogen surveillance in the environment, from where we retrieve relevant information.

2.2.2. Target hazards

The target hazards for which assays/methods used in pathogen surveillance in the environment are listed in table 8. Table 8 includes those pathogens pre-selected for the prioritisation exercise by the OH working group of EFSA.

Table 8. List of target hazards surveyed in the environment of those pre-selected for the prioritisation exercise by the OH working group of EFSA, according to the systematic review.

Virus		Bacteria		Protozoa		Helminth
Ebola virus disease virus		*Bacillus anthracis*		*Cryptosporidium spp.*		*Echinococcus multilocularis*
Hepatitis E virus		*Brucella (B. abortus, melitensis, suis, canis, ovis)*		*Giardia spp.*		*Echinococcus granulosus*
Influenza A virus (Avian)		*Coxiella burnetii*		*Leishmania spp.*		*Toxoplasma gondii*
Influenza A virus (Swine)		*Francisella tularensis*				
SARS-Coronavirus type 2		*Leptospira spp.*				
West Nile virus						

Other hazards surveyed in the environment alongside but not among those of the mentioned list are presented in Table 9.

The frequency of defined assays for the surveillance of pathogens, classified as virus, bacteria, protozoa, and helminths, in the environment was recorded and is shown below in Figure 18. The highest number of assays/methods on pathogen surveillance in the environment was done in viruses (49.4%), followed by bacteria (26.0%) and protozoa (22.4%).

A large percentage of available assays for the detection of bacteria (50.5%), viruses (38.2%) and helminths (37.5%) focus on hazards are not among those pre-selected for the prioritisation exercise by the OH working group of EFSA.

[4] https://doi.org/10.5281/zenodo.7409275

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Table 9. List of target hazards surveyed in the environment alongside but not among those pre-selected for the prioritisation exercise by the OH working group of EFSA, according to the systematic review.

Virus	Bacteria	Protozoa	Helminth
Ad helper virus	Bacteroidales-like microorganisms	Amoebae	*Ascaris spp.*
Adenovirus	*Balantidium coli*	*Acanthamoeba castellanii*	*Echinococcus (E. canadensis, ortleppi)*
Aichi virus	*Bartonella spp.*	*Blastocystis spp.*	*Toxocara spp.*
Avian Metapneumovirus	*Campylobacter spp.*	*Cyclospora cayetanensis*	*Trichuris spp.*
Barmah Forest virus	Coliforms	*Cystoisospora belli*	
Bovine coronavirus	*Cronobacter spp.*	*Dientamoeba fragilis*	
Bovine Viral Diarrhea virus	*Escherichia coli*	*Entamoeba histolytica*	
crAssphage	Fecal enterococci	*Entamoeba spp.*	
Emesvirus zinderi	*Francisella (F. mediasiatica, novicida, tularemia)*		
Enterovirus-G	*Helicobacter pylori*		
Hepatitis A virus	*Legionella spp.*		
Human Astrovirus	*Mycobacterium spp.*		
Human bocavirus	*Pseudomonas aerugionosa*		
Human coronavirus NL63	*Salmonella spp.*		
Human Parechovirus	*Shigella spp.*		
Human Polyomavirus	*Vibrio cholera*		
Infectious Human Enterovirus	*Yersinia enterocolitica*		
Influenza B virus			
Newcastle disease virus			
Norovirus			
Porcine coronavirus			
Respiratory Syncytial virus			
Rhinovirus			
Ross River virus			
Rotavirus			
Sapelovirus-A			
Sapovirus			
Teschovirus-A			
Tulane virus			

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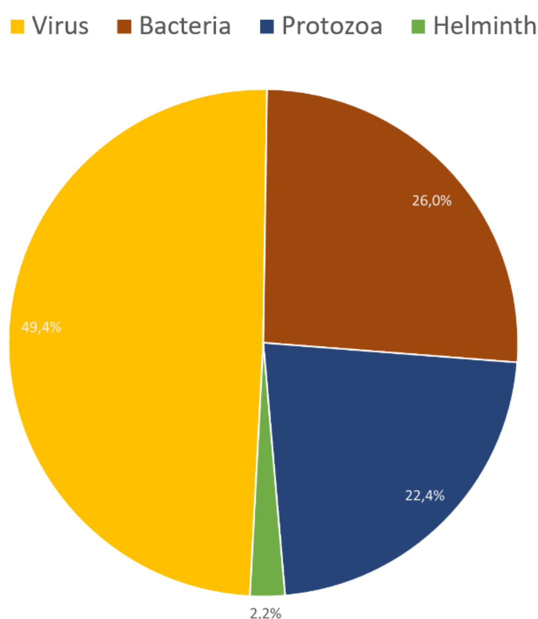

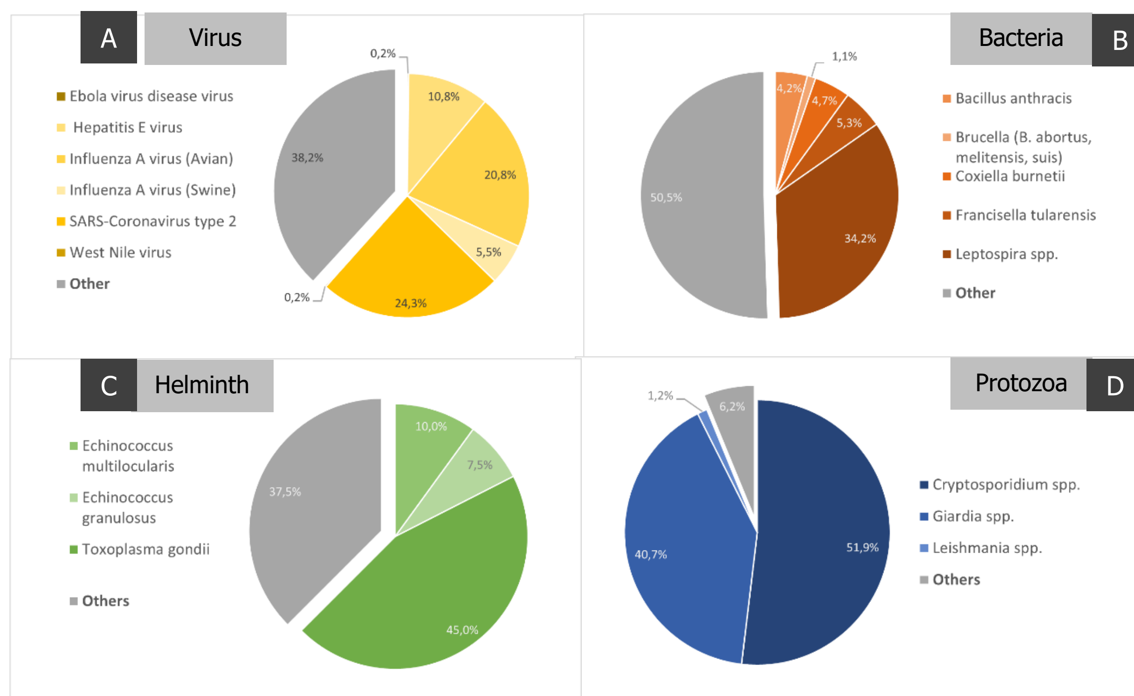

Figure 18. Sectoral graphs indicating the frequency (%) of defined assays for pathogens surveillance in the environment. Shown are those pathogens pre-selected for the prioritisation exercise by the OH working group of EFSA, as well as others found during the systematic review (grey). (A) Frequency (%) of defined assays for the surveillance of Viruses in the environment. The same information is shown for (B) Bacteria, (C) Helminths and (D) Protozoa.

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2.2.3. Type of environmental sample (matrix)

The type of environmental sample (matrix) used was recorded as: faecal, object surface, wastewater, water, air, sewage, soil and other. Water was most used matrix (25.4%) followed by object surface (16.6%), wastewater (16.3%), faecal (11.8%) and other samples to lesser extent (Figure 19).

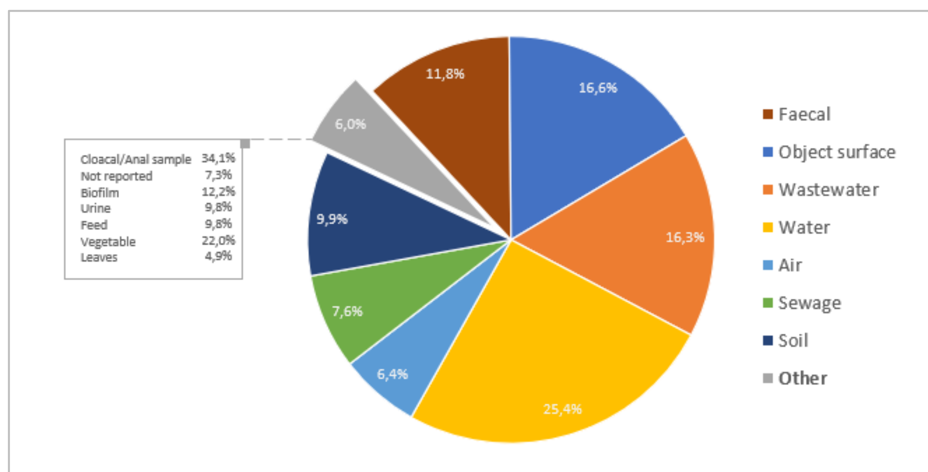

Figure 19. Frequency (%) of assays for pathogen surveillance in the environment by type of sample matrix.

Information was collected regarding the type of environment in which sampling was conducted. Three type environments were considered:

a) animal farm/market areas (*encapsules samples collected from animal markets, all sizes animal farms and agricultural facilities*);

b) urban areas (*meaning samples collected from humans, city and domestic settings as well as infrastructural facilities*) and;

c) natural areas (*samples collected from wild animals and environments*).

The information on type of environment in which sampling were more commonly surveyed for pathogens in the environment is presented in Figure 20.

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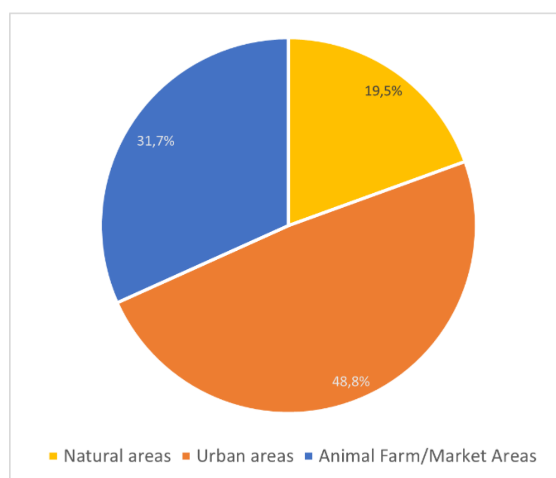

Figure 20. Frequency (%) of assays for pathogen surveillance in the environment by type of environment surveyed.

The frequency of defined assays by type of sample matrix as a function of their origin is shown in **Figure 21**. The most commonly sampled matrix in animal farm/market areas were object surfaces (33.3%). The wastewater was sampled to a higher extent in urban areas (32.8%) than other types of environmental sample, while 50% of defined assays applied in natural areas used water samples.

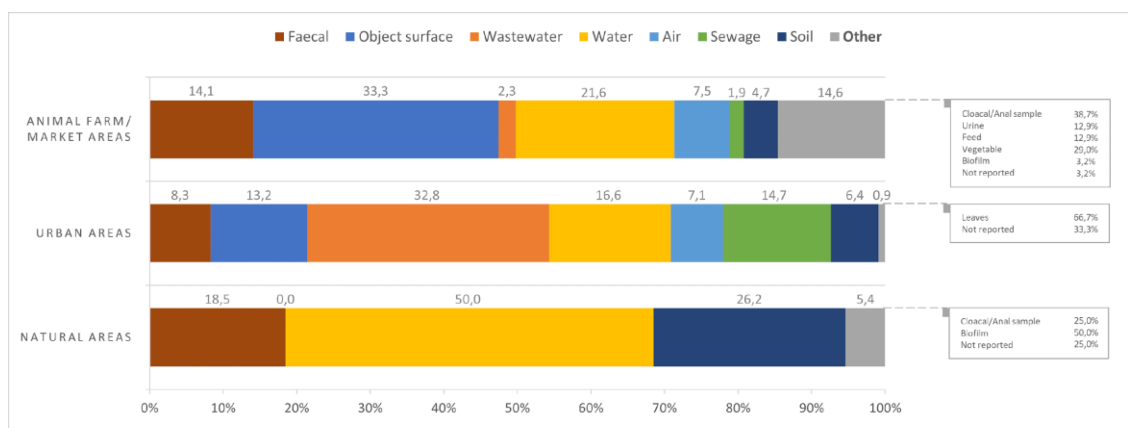

Figure 21. Frequency (%) of assays for surveillance of pathogens in the environment by type of sample matrix as a function of the environment in which the sampling was conducted.

Additionally, information on the different environmental matrices used to survey/detect a specific pathogen was analysed and presented in **Figure 22**. Wastewater was the most commonly used sample matrix for the surveillance of viruses in the environment (24.8%), closely followed by object surface (23.9%). To survey both bacteria or protozoa in the environment, water samples were the most often used (40.1% and 28.2%, respectively), while the search and detection of helminth pathogens in the environment was conducted in faecal (53.3%) and soil samples (40%) almost exclusively.

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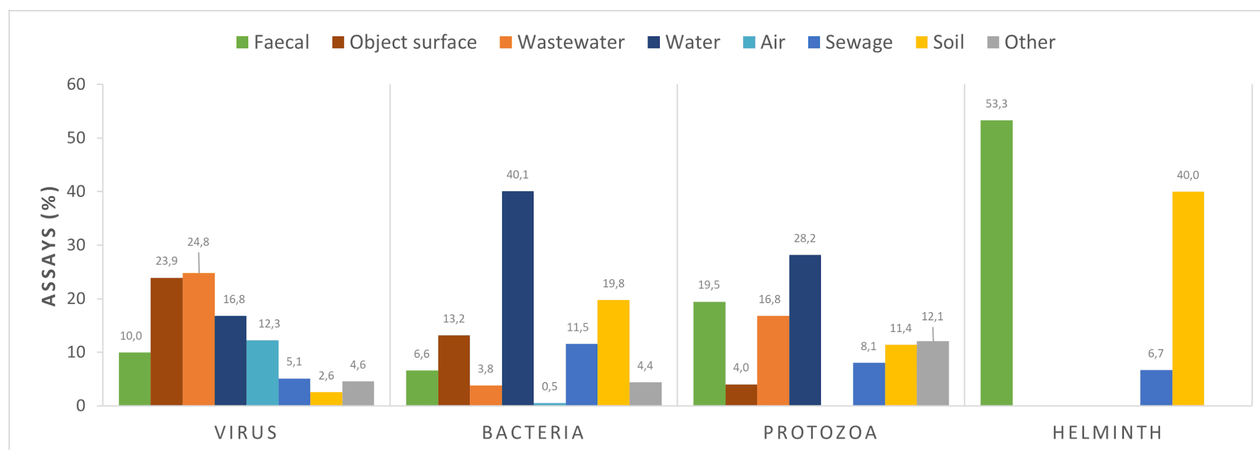

Figure 22. Frequency (%) of defined assays by type of sample matrix, as a function of the surveyed group of pathogens. Shown are those pathogens pre-selected for the prioritisation exercise by the OH working group of EFSA, as well as others found during the systematic review.

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2.2.4. Collection method

The methods used to collect samples from the environment were recorded as:

a) swab;

b) grab sample;

c) automatic sampling; and

d) other methods.

The variation of the frequency of methods used to collect samples in the different assays, as well as those assays to which no method of collection was described, is presented in Figure 23.

In general consideration, grab sample was the most commonly used collection method, since it was used in 41.1% of the analysed assays. A total of 28.5% of assays did not report or describe their collection method of choice.

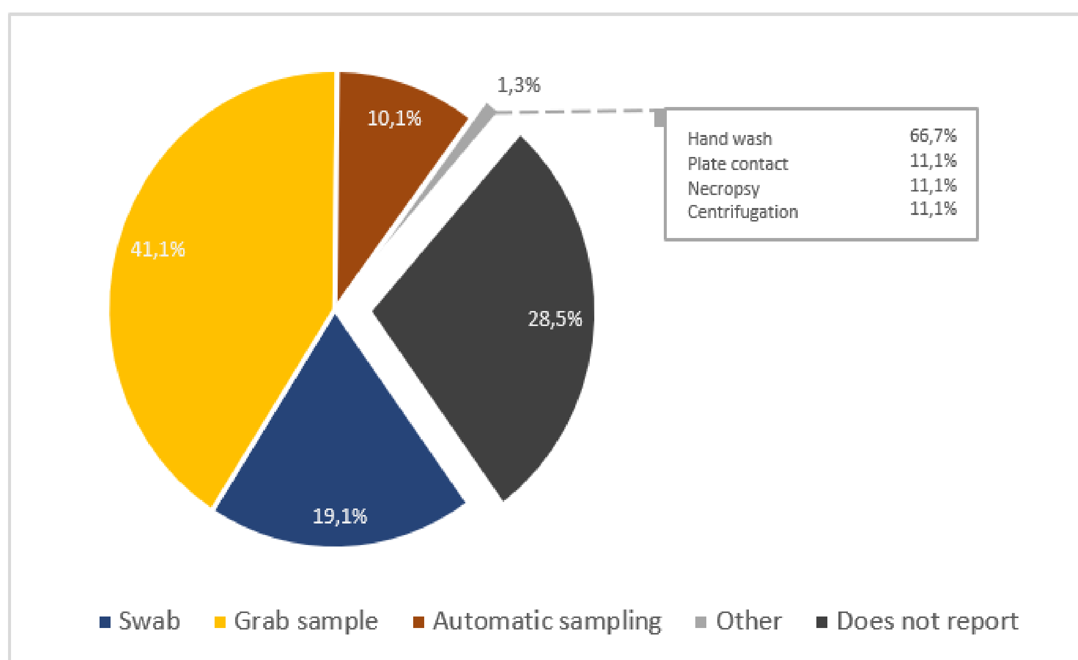

Figure 23. Frequency (%) of defined assays for the surveillance of pathogens in the environment by sampling collection method.

Information was extracted on the different methods used to collect different environmental matrices and presented in Figure 24. Most sampling of faecal (62.7%), wastewater (81%), water (92.2%), sewage and soil (both 91.4%) and other, less common samples (61.1%), was conducted through the collection of a grab sample. Object surfaces were sampled most commonly through swabbing (90.3%), while air was sampled automatically, exclusively.

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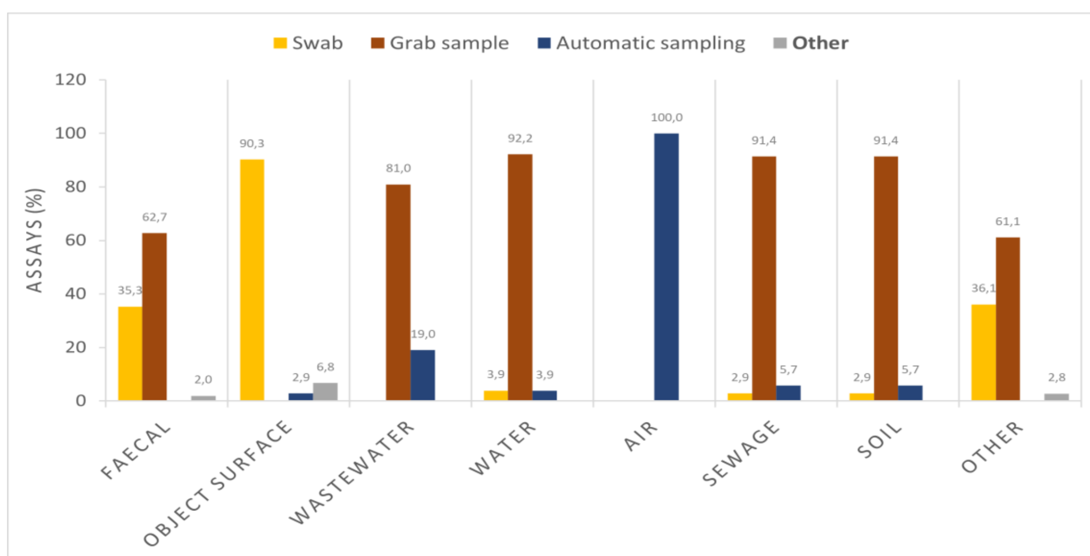

Figure 24. Frequency (%) of defined assays by sampling collection method as a function of the type of sample collected.

2.2.5. Pre-analytical treatment (sample)

From the analysed assays, information was assessed regarding the steps of sample treatment aimed at improving downstream pathogen detection and identification. The considered sample treatment were grouped in:

a) storage;
b) sample concentration;
c) pathogen isolation;
d) inhibitor search/removal; and
e) others.

Storage

Of the analysed assays, 46.4% gave description of long-term (equal or over 24h) sample storage conditions (Figure 25).

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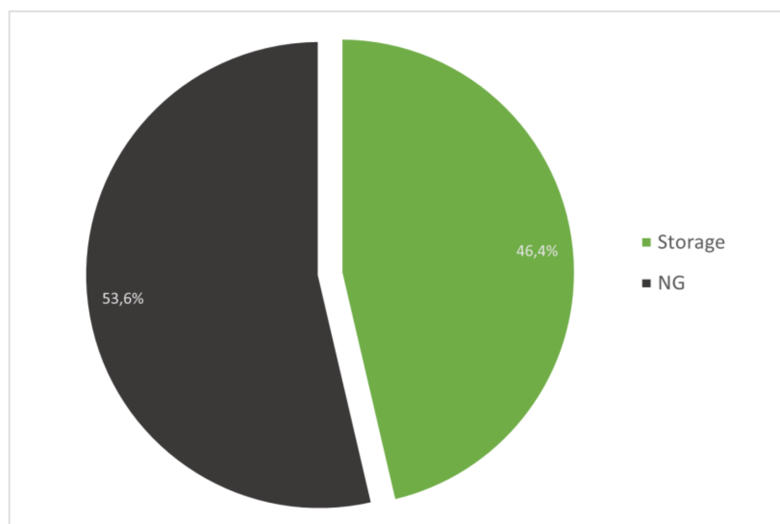

Figure 25. Frequency (%) of analysed assays, which give (Storage) or do not give (NG) information on (long-term) sample storage conditions.

The information on the recorded storage conditions by sample matrix type is presented in figure 95. The information on storage conditions was grouped in the following categories:

a) room temperature;
b) refrigerated;
c) frozen (-20ºC);
d) frozen (-80ºC); and
e) others, including, but not restricted to, the flash-freezing technique (-70ºC).

In general, refrigeration was the primary form of storage of environmental samples on record, excluding air sampling, to which freezing at -80ºC followed sampling for 61.9% of included assays (Figure 26). Nevertheless, in some assay's samples were left at room temperature prior to analysis.

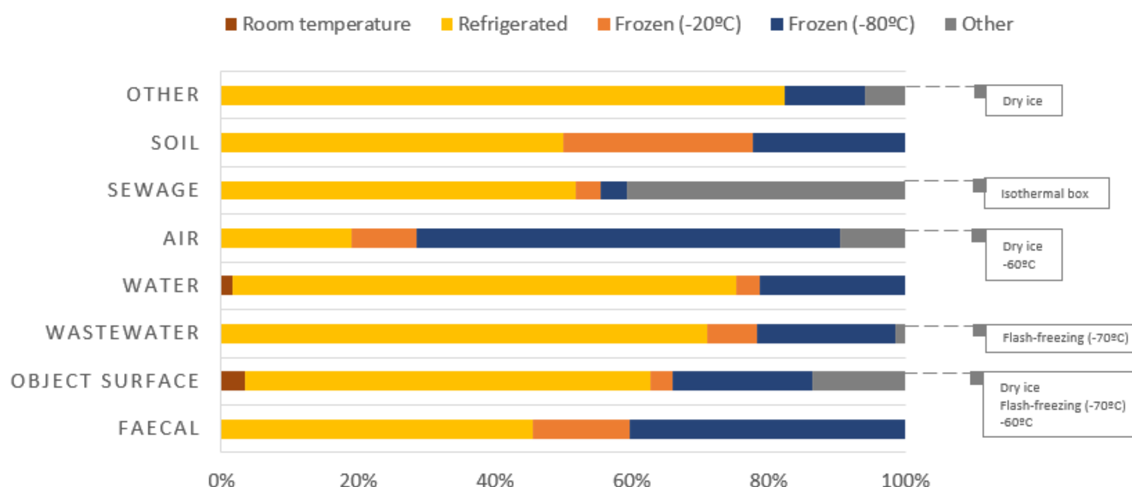

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Figure 26. Frequency (%) of the analysed assays organized by group of storage conditions by type of sample matrix.

The frequency variation of used storage conditions according to the group type of pathogens is represented in Figure 27.

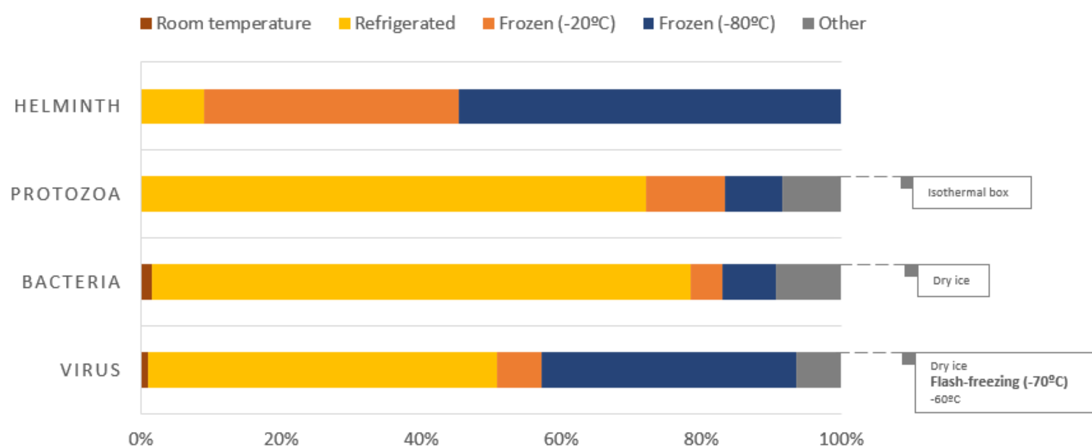

Figure 27. Variation of the frequency (%) of assays using the different sample storage conditions by group type of pathogens.

When identifying Bacteria and protozoa in the assays, authors most commonly refrigerate the collected environmental non-invasive samples, while for the surveillance of helminths and viruses, frozen (-20 and -80ºC) is the most common storage condition method. It should be notice that the flash-freezing technique (-70ºC) is used only for the storage of samples in virus and bacteria focused assays.

Sample concentration

In general, water-based samples seem to be the most common matrix undergoing pre-analytical concentration, with 81.3% and 71.3% of assays analysing wastewater and water, respectively, concentrating these samples prior to pathogen detection. Assays analysing soil (51.5%), faecal (37%) and sewage (36.5%) also report sample concentration. This information is given below in Figure 28.

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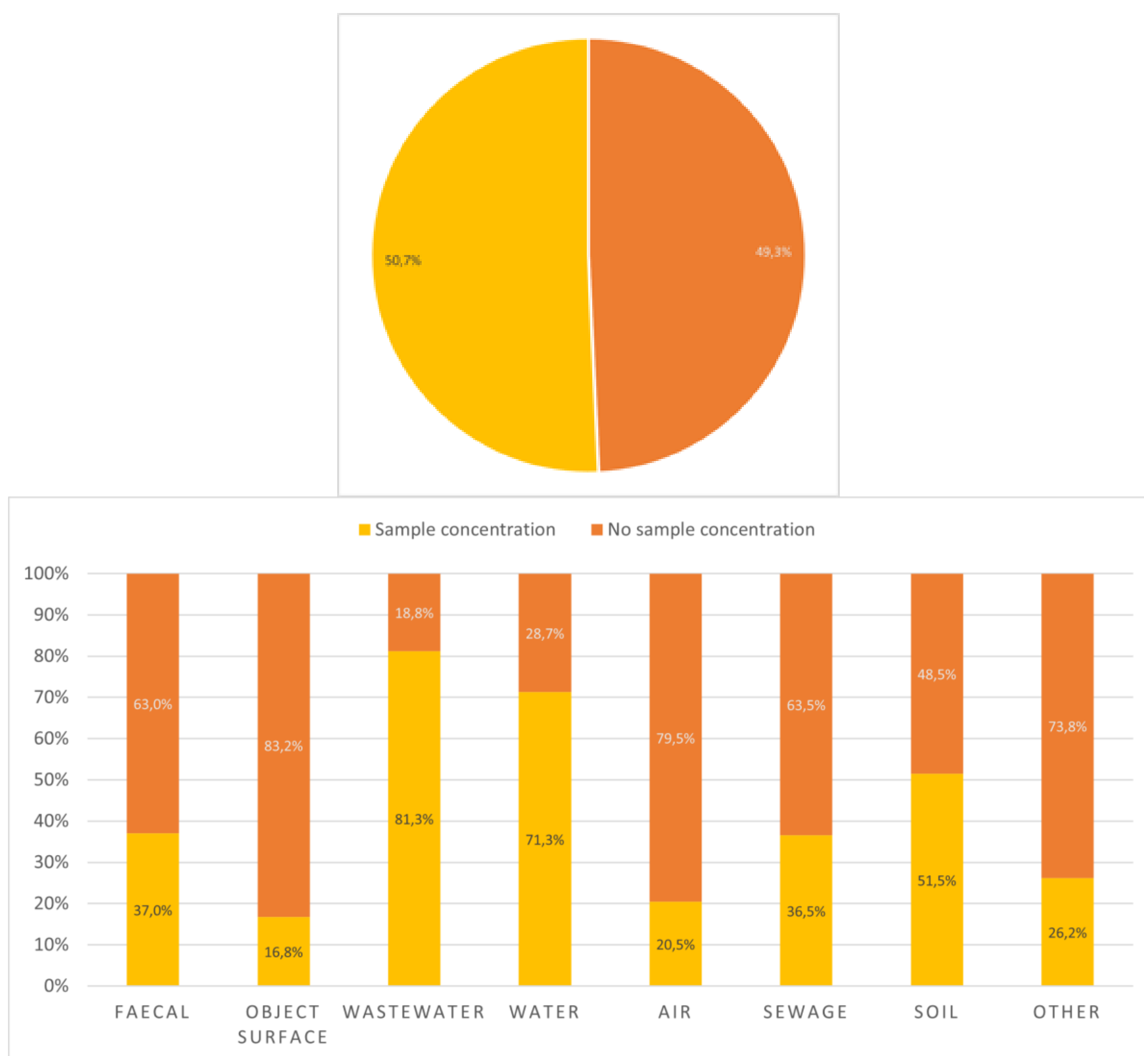

Figure 28. The sectoral graph (right) shows the frequency (%) of total assays which include or do not include a pre-analytical treatment step of sample concentration. The same information is given by type of sample matrix (left).

Inhibitor search/removal

Considering the prevalent presence of possible reaction inhibitors in environmental samples, information regarding the search and/or removal of these prior to sample analysis was recorded as one of the defined pre-analytical sample treatments.

Search of the literature revealed a concern for possible inhibitors mainly in assays employing molecular methods of pathogen detection and identification, namely in nucleic acid-based based methods (14.9%) and in immunology-based methods (7.3%). This information is displayed in Figure 29.

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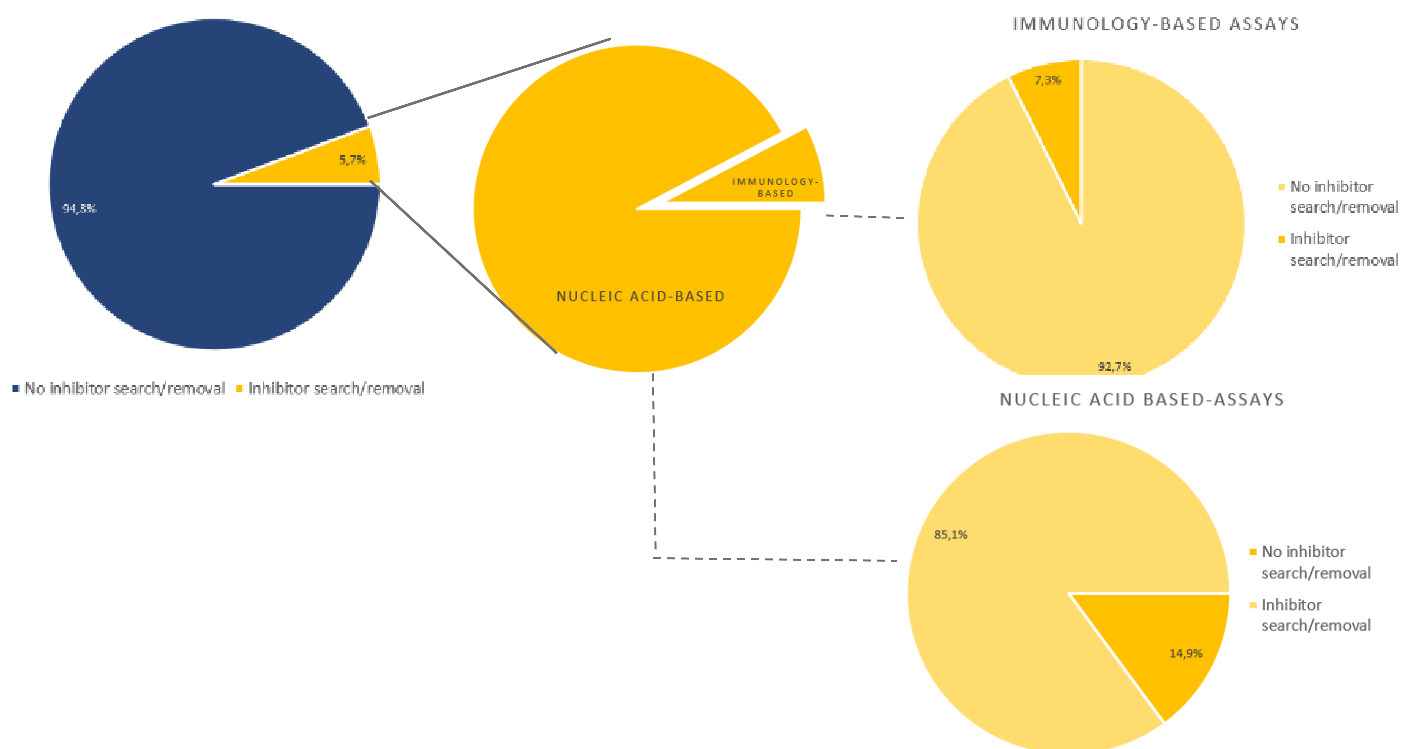

Figure 29. Frequency (%) of defined assays employing pre-analytical inhibitor search/removal steps.

More detailed information on the inhibitor search/removal methods was recorded and summarized in Table 10.

Of the included methods, the wash and usage of a commercial kit used for the removal of inhibitors prior to sample analysis, while the pre-analytical spiking of samples and inclusion of an internal control or serial dilutions were used to search for inhibitors or any inhibitory action. The spiking/Internal control was the most common inhibitor search/removal used method.

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Table 10. List of recorded methods for inhibitor search/removal. Given is application as it pertains to sample matrix, downstream pathogen detection method and target hazard, as well as their frequency (%) among those assays employing pre-analytical inhibitor search/removal steps.

Inhibitor search/ removal method	Sample matrix	Detection method	Target hazard	Assay frequency (%)
Commertial kit	Water Wastewater	Nucleic acid-based	Bacteria Protozoa Virus	9.6%
Serial dilutions	Object surface Water Faecal Wastewater	Nucleic acid-based	Virus Protozoa Helminths	21.3%
Spiking/Internal control	Faecal Object surface Wastewater Water Sewage Soil Other	Nucleic acid-based Immunology-based	Bacteria Protozoa Virus	63.8%
Wash (deinized, distilled water/chloroform)	Faecal Soil Water	Nucleic acid-based Immunology-based	Bacteria Protozoa Virus	5.3%

Of the included methods, the wash and usage of a commercial kit used for the removal of inhibitors prior to sample analysis, while the pre-analytical spiking of samples and inclusion of an internal control or serial dilutions were used to search for inhibitors or any inhibitory action. The spiking/Internal control was the most common inhibitor search/removal method.

2.2.6. Pathogen detection, discrimination, and identification

The nucleic-acid-based methods are the most commonly used approach for pathogen detection, identification, and characterization (Figure 30). A detailed list of pathogen identification methods used in the analysed assays, considering the different group pathogens, is presented in the Supplementary Tables 1, 2, 3, 4 and 5. In these supplementary tables are presented the list of pathogen identification methods recorded in the analysed assays (full name and respective abbreviation), as well as, for each group of pathogens (Bacteria, Virus, Protozoa and Helminths), the detection methods are organized by techniques, pre-analytical treatment strategies, type of sample matrix and target hazard, according the method category (Nucleic acid-based; Immunology-based; Culture and Biochemistry-based; Others).

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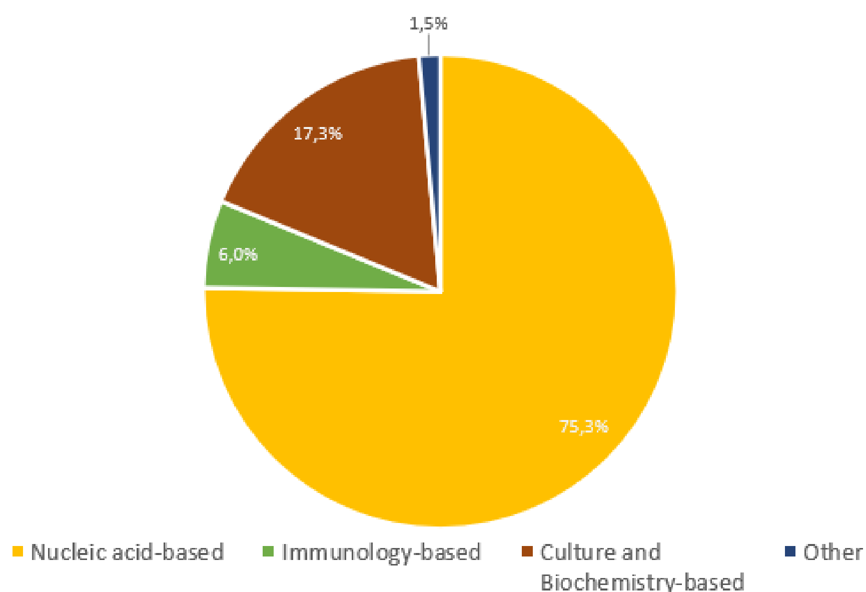

Figure 30. Frequency (%) of analysed assays for pathogen surveillance in the environment by pathogen identification method category.

Table 11 shows the list of pathogen identification methods from included assays. Given is the full name and respective abbreviation, when called for, of the method variants from each defined method category.

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Table 11. List of pathogen identification methods from included assays. Given is the full name and respective abbreviation, when called for, of the method variants from each defined method category.

Abbreviation	Name
Nucleic acid-based Methods	
B1-LF-RPA	Recombinase Polymerase Amplification of the B1 gene visualized by a Lateral Flow strip
Conventional PCR	Conventional Polymerase Chain Reaction
	CRISPR/Cas12a
ddPCR	drop digital PCR
DNA-AuNP probe assay	Deoxyribonucleic acid-Gold NanoParticle probe assay
ICC-qPCR	Integrated Cell Culture quantitative PCR;
IR-NAAS	Interference Reduction Nucleic Acid Amplification Strategy
LAMP	Loop-mediated isothermal Amplification
MFqPCR	Microfluidic quantitative PCR
MRT-PCR	Multpilex Real-Time PCR
Multiplex qPCR	Multiplex quantitative-PCR
Nested RT-PCR	Nested Real-Time PCR
NGS	Next Generation Sequencing
PCR-RFLP	Restriction Fragment Length Polymorphism PCR
qPCR	quantitative-PCR
rRT-PCR	Real-Time reverse transcription PCR
rRT-qPCR	Real-Time quantitative reverse transcription PCR
RT-ddPCR	Real-Time drop digital PCR
RT-LAMP	Real-Time LAMP
RT-PCR	Real-Time PCR
RT-qPCR	Real-Time quantitative PCR
RV-PCR	Rapid Viability PCR
RV-RT-PCR	Rapid Viability Real-Time PCR
Sanger sequencing	
SNP-RT-PCR	Single Nuclear Polymorphisms Real-Time PCR
Immunology-based Methods	
DFA	Direct Immunofluorescence Assay;
EIA	Enzime Immunoassay
ELISA	Enzyme-Linked Immunosorbent Assay
HA	Hemagglutinating test
HI	Hemagglutination Innhibition test
IFA	Immunofluorescence Assay

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IFAT	Quantitative Immunofluorescence Assay
IMS-IFA	Immunomagnetic Separation – IFA
Latex Agglutination Test	
MAT	Microscopic Agglutination Test;
Culture and Biochemistry-based Methods	
	API 20E Strip test
	Biotyping
	Chromogenic Substract technique
	Colilert test
	Count Plates
	Cytochrome c Oxidase test
	Darkfield microscopy
	Epifluorescence Microscopy
	Flotation technique
	Fluorescence Microscopy
	Gram staining
	Hippurate Hydrolysis test
	Light Microscopy
	Microbiologic Culture
	MicroScan system
MPN	Most Probable Number technique
	Neubauer technique
	Paper-based Eletrochemical Quantification
	Slide Agglutination test
	Ziehl-Neelsen staining
Other	
	Flow cytometry
	Fluorometry
	Radiometric colorimetric and AgNPs-fluorescence dual mode sensing
MALDI-TOF-MS	Matrix-Assisted Laser Desorption/Ionization-Time Of Flight-Mass Spectrometry

Among the DNA/RNA based pathogen identification methods, the ones using real time PCR and quantitative PCR (RT-qPCR, RT-PCR, qPCR and rRT-PCR), are the most common used (Figure 31a).

When considering the culture and biochemistry-based approach for pathogen identification, the microbiologic culture (33.6%) is the most commonly used method (Figure 31b).

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Regarding the immunology-based approaches, the HA (22.5%), IFA (20%) and IFAT (17.5%), are the most used methods for detecting and identifying the pathogens (Figure 32a).

Among the other methods used, the flow cytometry is the most common used method (Figure 32b).

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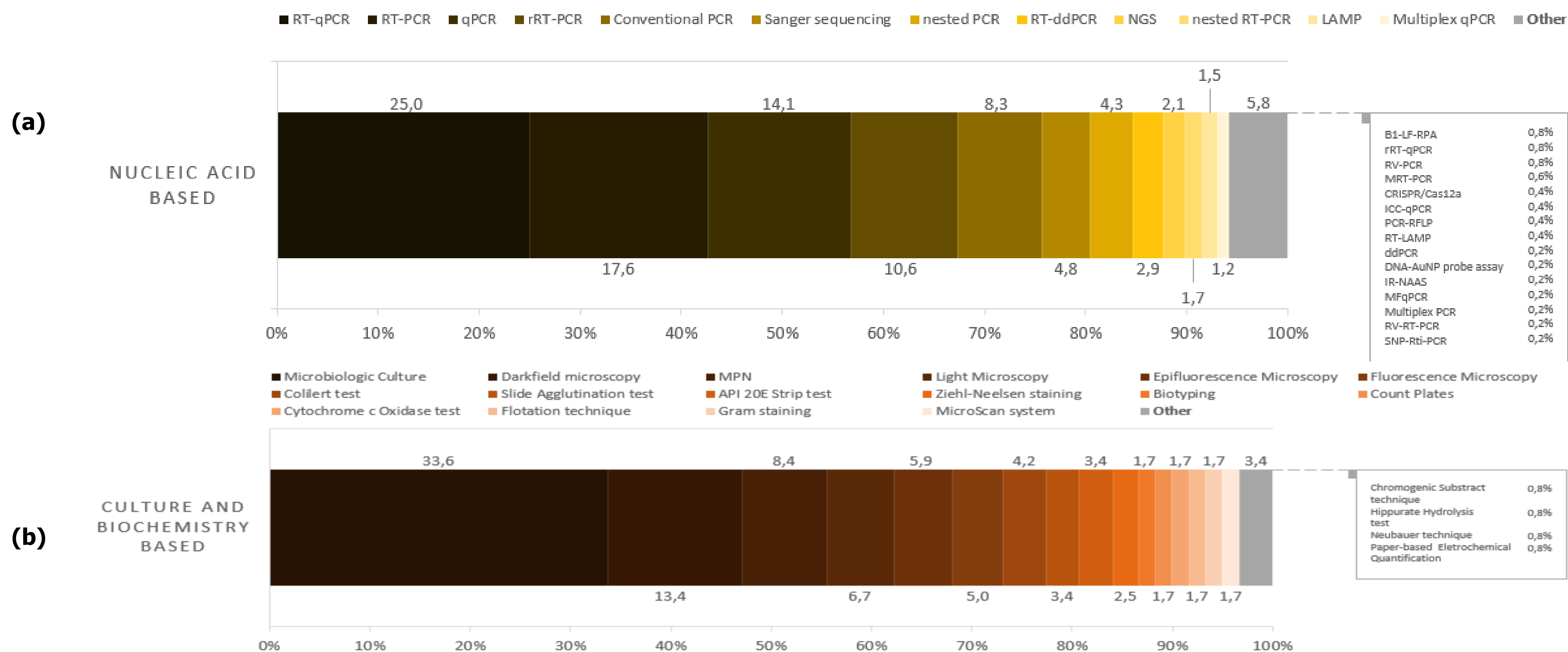

Figure 31. (a) Frequency (%) of defined assays employing a DNA/RNA based Pathogen Identification Method for the surveillance of pathogens in the environment by Method Variant. (b) Frequency (%) of defined assays employing a DNA/RNA based Pathogen Identification Method for the surveillance of pathogens in the environment by Method Variant.

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(a)

■ HA ■ IFA ■ IFAT ■ DFA ■ HI ■ MAT ■ EIA ■ ELISA ■ Ether-IMS-IFA ■ IMS-IFA ■ Latex Agglutination Test ■ NaPP-IMS-IFA

IMMUNOLOGY BASED

22,5 20 17,5 10 10 5 2,5 2,5 2,5 2,5 2,5 2,5

0% 10% 20% 30% 40% 50% 60% 70% 80% 90% 100%

(b)

■ Flow cytometry ■ Radiometric colorimetric and AgNPs-fluorescence dual mode sensing ■ Fluorometry ■ MALDI-TOF-MS

OTHER

50,0 30,0 10,0 10,0

0% 10% 20% 30% 40% 50% 60% 70% 80% 90% 100%

Figure 32. (a) The frequency (%) of defined assays employing a Protein based Pathogen Identification Method for the surveillance of pathogens in the environment by Method Variant. (b) Frequency (%) of defined assays employing Other Pathogen Identification Methods for the surveillance of pathogens in the environment by Method Variant.

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Although the nucleid-acid based approach is the most commonly used in pathogen identification and characterisation, its frequency, as well as the other approaches, varies when considering the different pathogens groups (Figure 102). While the nucleid-acid based methods are the ones used in over 90% of virus studies, in Bacteria this frequency decreases to 54%, while the culture and biochemistry-based methods increase to 42% (Figure 33).

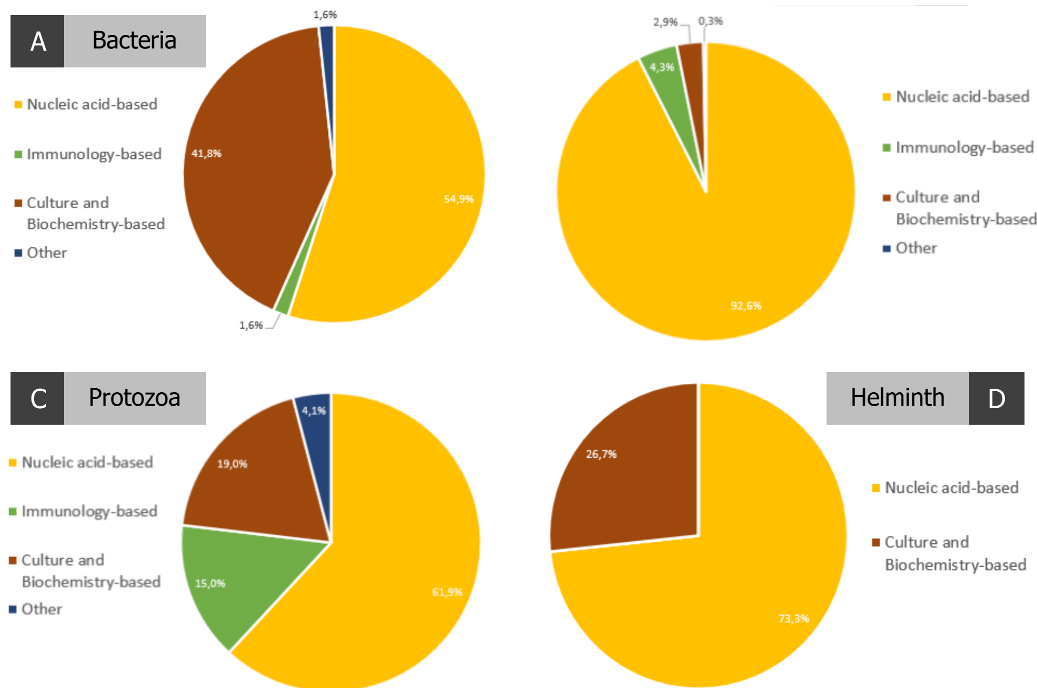

Figure 33. Frequency (%) of defined assays by pathogen identification method category (Nucleic acid-based, Culture and Biochemistry-based, Immunology-based and Others) as a function of the surveyed type pathogen: (A) Bacteria, (B) Virus, (C) Protozoa and (D) Helminth.

The nucleic-acid based approach is, in general, the one greatly used when considering the different types of non-invasive samples (Figure 34). Nevertheless, it should be noticed that when using sewage non-invasive samples, the culture and biochemistry-based methods are used in 52% of the analysed assays (Figure 34).

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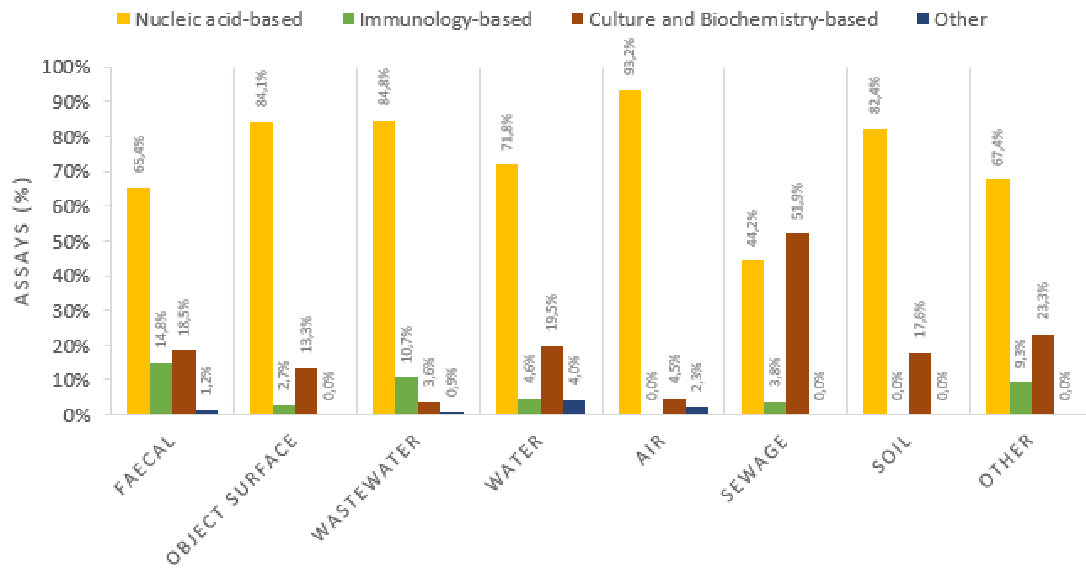

Figure 34. Frequency (%) of defined assays by category of pathogen identification method as a function of the type of sample collected.

The frequency of used nucleic-acid-based methods varies across the different types of the used non-invasive samples (Figure 36). While real time PCR is the one most used in general, in sewage and other types of samples, the normal PCR is the one mostly used (Figure 35).

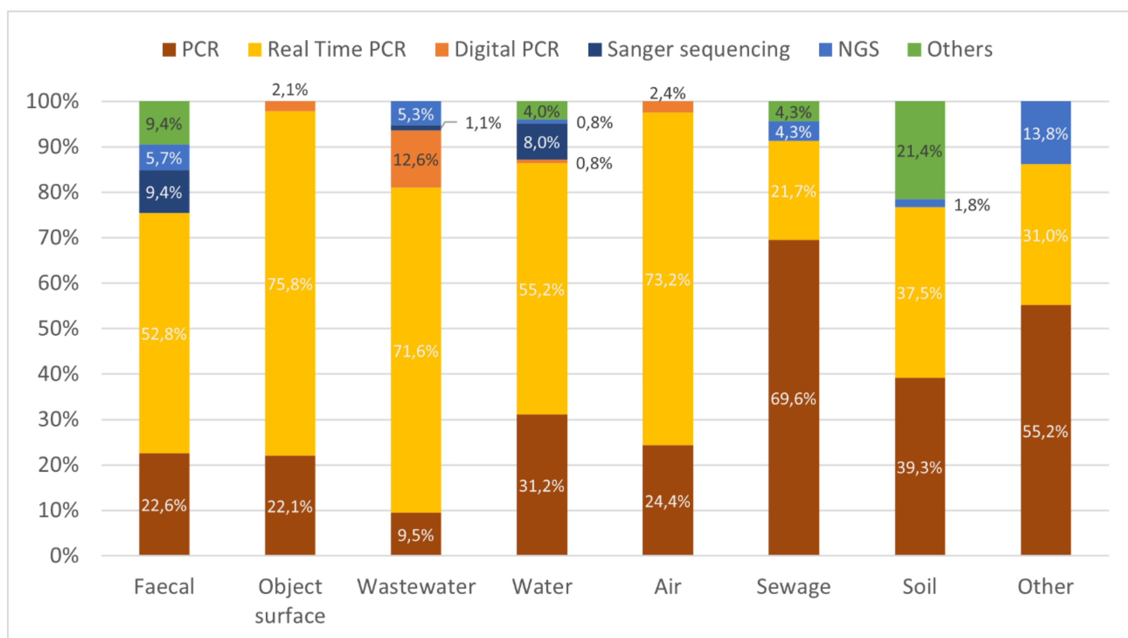

Figure 35. Frequency (%) of defined assays employing DNA/RNA based methods for the surveillance of pathogens in the environment by method variant as a function of the type of analysed sample. "Others" include method variants such as LAMP (Faecal, Water and Other

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samples), IR-NAAS (Sewage samples), CRISPR/Cas12a (Soil samples), the DNA-AuNP probe assay (Water samples), PCR-RFLP (Faecal samples) and the B1-LF-RPA assay (Water and Soil samples).

When considering the Protein based methods for the surveillance of pathogens in the environment, used in the different sample types, the one more commonly utilized is the Immunofluorescence (Figure 36). Nevertheless, it should be noticed that there is a high variation, which can also be based in the lower number of analysed assays, since most of them use the nucleic-acid based approaches.

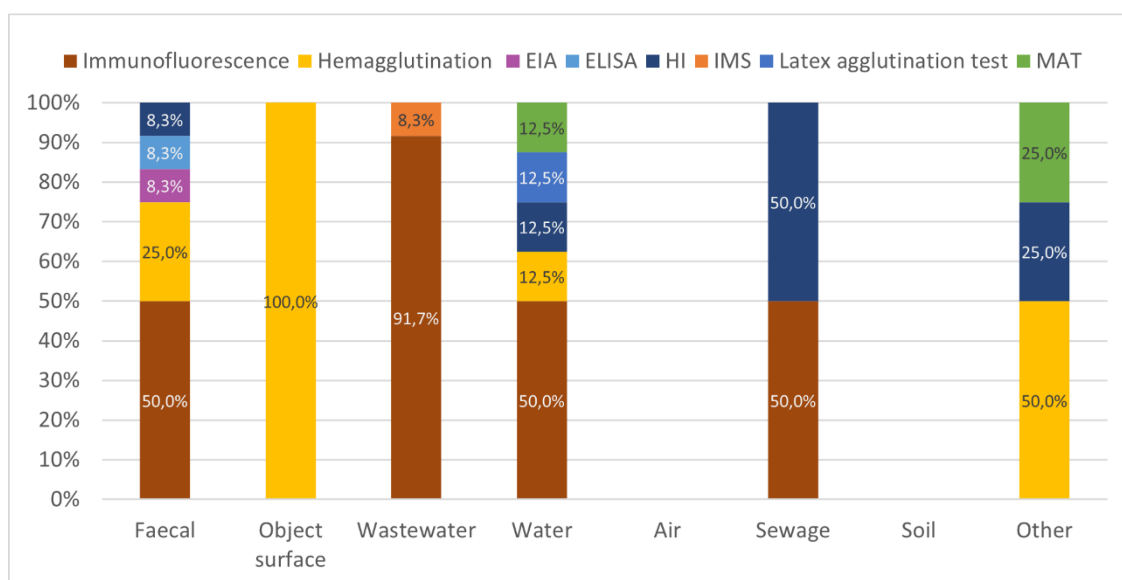

Figure 36. Frequency (%) of analysed assays employing Protein based methods for the surveillance of pathogens in the environment by method variant as a function of the type of analysed sample.

The frequency variation of the analysed assays using biochemistry-based methods for the pathogen surveillance in the environment by method variant as a function of the type of analysed sample is presented in Figure 37. It can be shown that there is a high variability on the used methods according to the different types of samples. Nevertheless, there is a tendency on the use of microbiologic cultures.

Figure 38 shows the frequency (%) of defined assays employing Biochemistry based methods for the surveillance of pathogens in the environment by method variant as a function of the type of analysed sample.

The variation in the frequency of the analysed assays employing DNA/RNA based methods for the pathogen surveillance in the environment by method variant as a function of the type pathogen identified is presented in Figure 39. It is clear to observe that in all pathogen groups the Real time PCR method is the one most commonly used, varying from 43.2% in Protozoa to 90.9 in Helminths.

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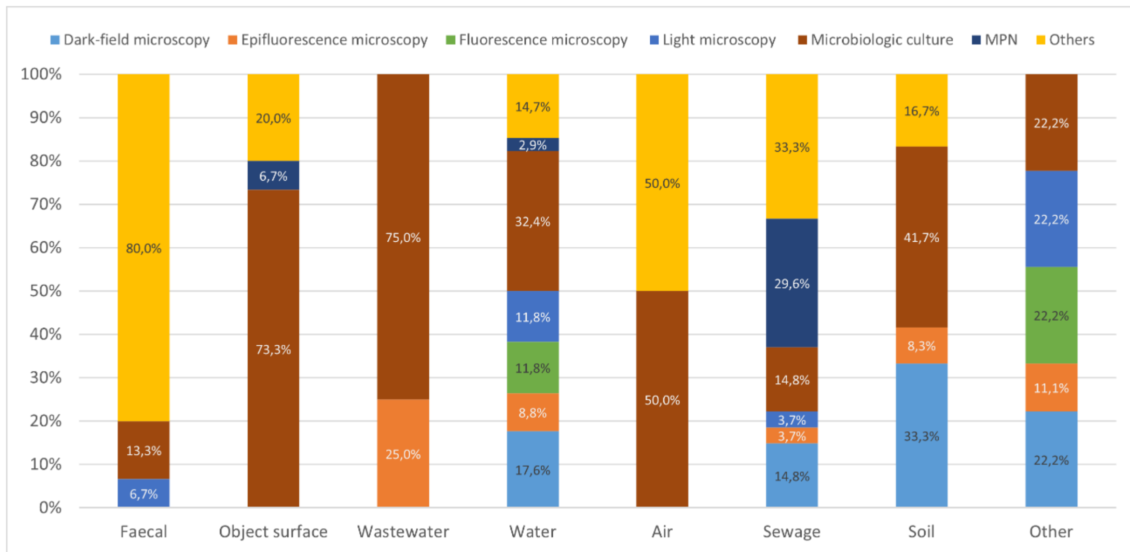

Figure 37. Frequency (%) of defined assays employing Biochemistry based methods for the surveillance of pathogens in the environment by method variant as a function of the type of analysed sample. "Others" include method variants such as API 20E Strip test Biotyping (Sewage samples), Colilert test (Object surface, Water and Soil samples), Count plates (Object surface samples), Chromogenic substrate technique (Water samples), cytochrome C oxidase test (Faecal samples), Flotation technique (Faecal samples), Gram staining test (Faecal samples), hippurate hydrolysis test (Faecal samples), MicroScan system (Faecal samples), Neubauer technique (Faecal samples), Paper-based eletrochemical quantification (Air samples), Slide agglutination (Sewage samples) and Ziehl-Neelsen staining (Faecal, Water and Soil samples).

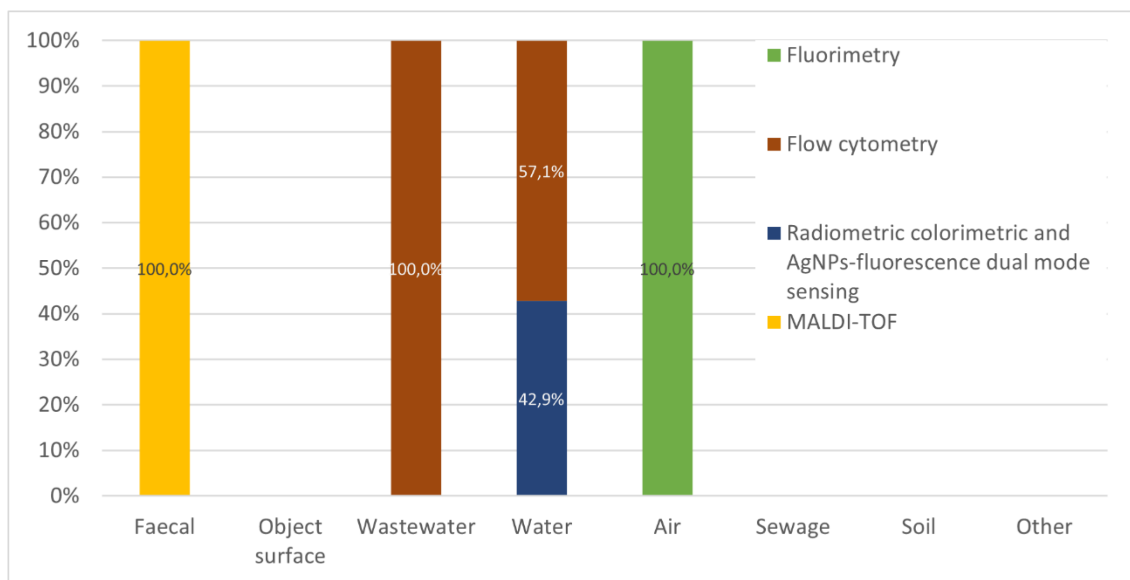

Figure 38. Frequency (%) of defined assays employing Biochemistry-based methods for the surveillance of pathogens in the environment by method variant as a function of the type of analysed sample.

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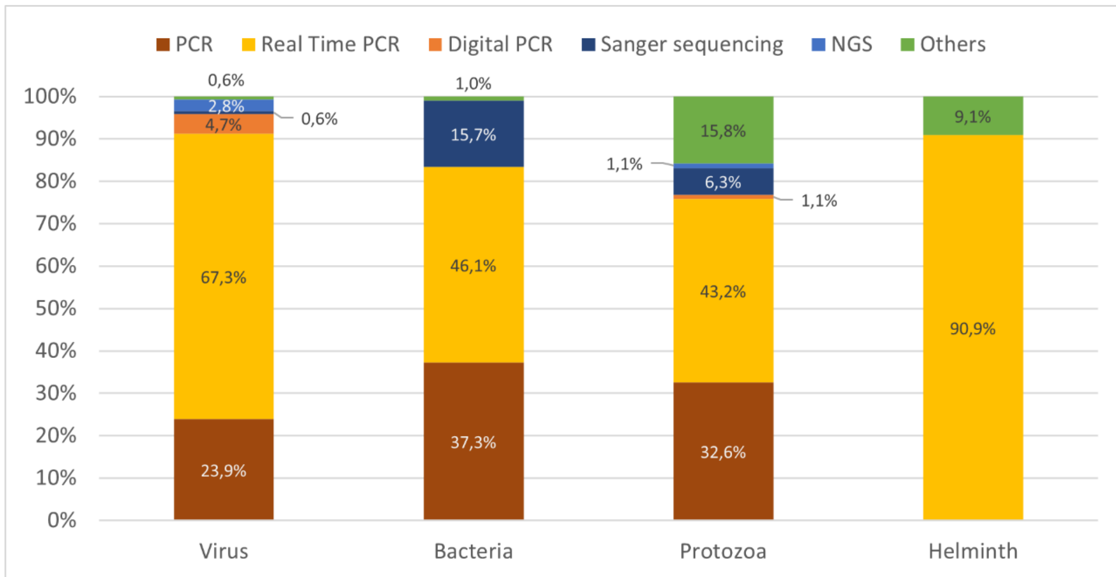

Figure 39. Frequency (%) of analysed assays employing DNA/RNA based methods for the pathogen surveillance in the environment by method variant as a function of the type pathogen identified. "Others" include method variants such as LAMP (Protozoa, Helminth and Virus), IR-NAAS (Virus), CRISPR/Cas12a (Protozoa), the DNA-AuNP probe assay (Bacteria), PCR-RFLP (Protozoa) and the B1-LF-RPA assay (Protozoa).

When considering the variation of the frequency of analysed assays that used protein-based method in pathogen surveillance in environmental samples by method variant, we can see differences among the group type of pathogens (Figure 40). While in Viruses the most used is Hemagglutination, MAT is the one more commonly used in bacteria and the immunofluorescence in Protozoa (Figure 40).

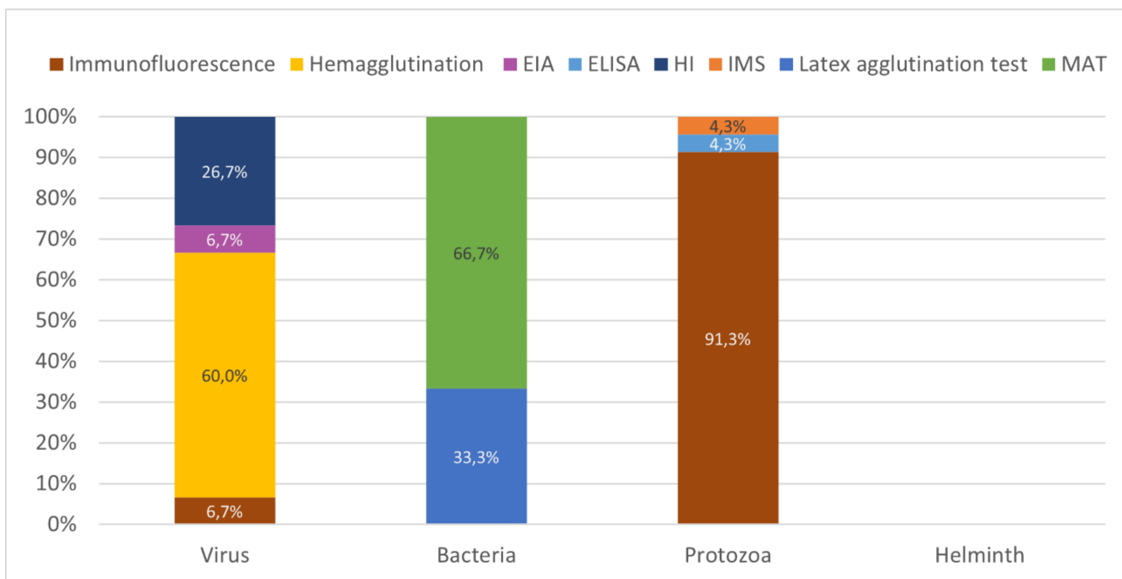

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Figure 40. Frequency (%) of defined assays employing Protein based methods for the surveillance of pathogens in the environment by method variant as a function of the type pathogen identified.

In the analysed assays that employed Biochemistry based methods for pathogen surveillance in the environment by method variant, the Microbiologic culture was the most common methods used in Virus and Bacteria, while light microscopy in Helminths (Figure 41). In the protozoa the most commonly used method was the Epifluorescence microscopy.

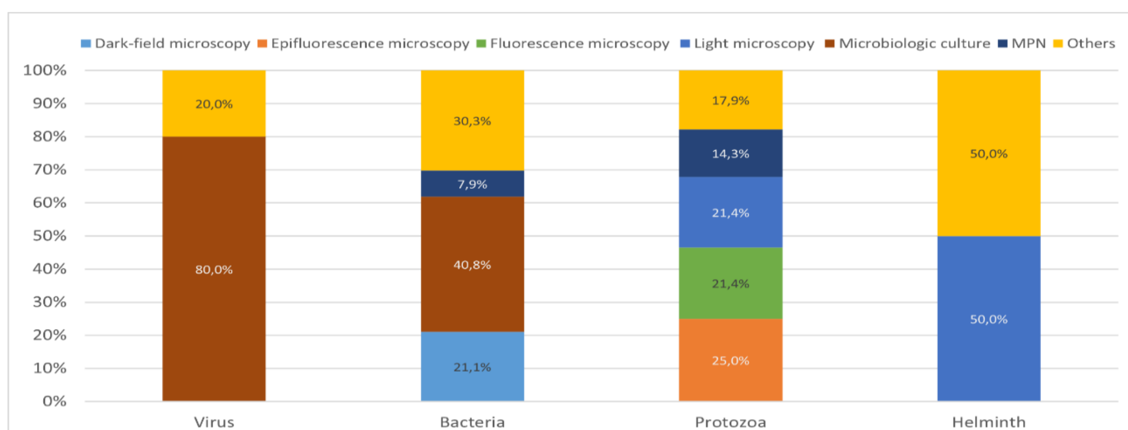

Figure 41. Frequency (%) of analysed assays employing Biochemistry based methods for pathogen surveillance in the environment by method variant as a function of the type pathogen identified. "Others" include method variants such as API 20E Strip test (Bacteria), Biotyping (Bacteria), Colilert test (Bacteria), Count plates (Virus), Chromogenic substrate technique (Protozoa), cytochrome C oxidase test (Bacteria), Flotation technique (Helminth), Gram staining test (Bacteria), hippurate hydrolysis test (Bacteria), MicroScan system (Bacteria), Neubauer technique (Protozoa), Paper-based electrochemical quantification (Bacteria), Slide agglutination (Bacteria) and Ziehl-Neelsen staining (Protozoa).

Finally, the variation of the analysed assays employing Protein based methods for pathogen surveillance in the environment by method variant was quite different depending on the group type of pathogens (Figure 42). While in Virus the fluorimetry was used in 100% of the cases, in bacteria it was the radiometric colorimetric and AgNPs-fluorescence dual mode sensing (100%) and in the Protozoa the flow cytometry (83%). Nevertheless, this variation should be analysed with cautions due the low number of analysed assays.

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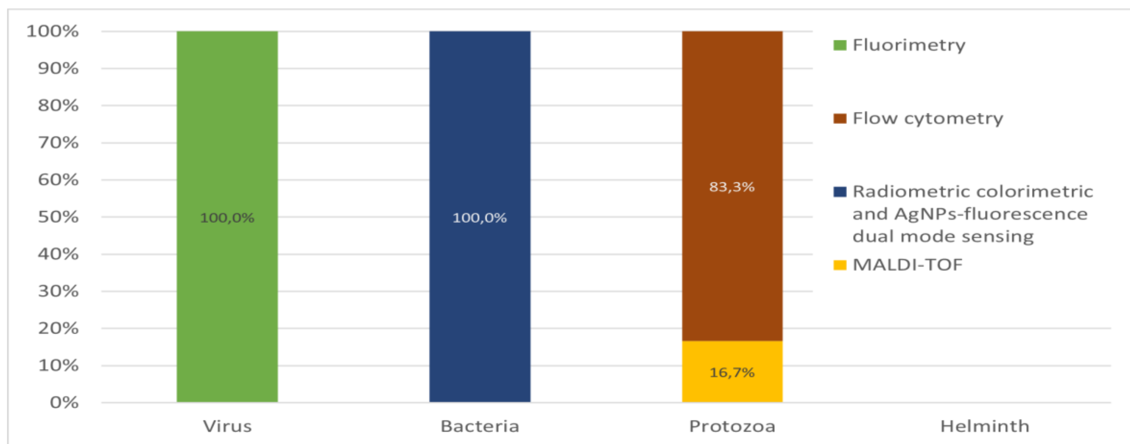

Figure 42. Frequency (%) of defined assays employing Protein based methods for the surveillance of pathogens in the environment by method variant as a function of the type pathogen identified.

2.3. Discussion

After a preliminary search using the defined strings, we were able to retrieve 1253 publications. However, after limiting our search with the inclusion and exclusion criteria we retrieved 191 publications. After reading and analysing these 191 publications, we were able to consider a total of 686 assays for pathogen surveillance in the environment, from where we retrieve relevant information.

A large percentage of available assays for the detection and surveillance of pathogens in the environment focus on hazards not among those pre-selected for the prioritisation exercise by the OH working group of EFSA. However, these may be a source of new, untested, methods for the surveillance of those listed pathogens of higher epidemiological importance.

2.3.1. Type of environmental sample (matrix)

Almost half of the recorded assays for the detection of pathogens in the environment were applied and tested for surveillance in urban areas. Such is comprehensible considering the higher epidemiological and health risks for the population and the recent pandemic state, which caused a boom in epidemiological studies in populated areas. The same could be said to explain the higher frequency of assays implemented for the surveillance of animal farm/market areas, given their connection to food and health safety.

Nevertheless, the less disturbed areas, the natural and wild environments may hold undiscovered and potentially epidemiologically significant hazards and hosts and should receive higher attention. Natural areas, however, may represent different challenges for pathogen detection and identification, namely, sample availability, pathogen prevalence and inhibitors. The existing assays/methods, developed for urban and farm areas, should be tested, and applied for the surveillance of natural areas.

In the studies of animal farm/market areas the object surface and water are the most common sample matrices. Object surface sampling can be focused, searching for points of presumed higher pathogen prevalence (contact transmission points, animal living areas EX cages).

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Nevertheless, pathogen surveillance in water (drinkers and watering systems) permits the early detection and treatment of possible outbursts.

In urban areas, the wastewater has many possible contamination sources, giving a complex image of the environment/population. But it can be used for early detection within populated areas (as well as sewage). The development and optimization of assays for the detection of hazards in wastewater may help better control wastewater treatment and health safety.

In natural areas, the non-invasive sampling of wildlife faeces can give information on possible hosts/new hosts, vectors, and emerging epidemics. Moreover, despite a very complex sample matrix, water can be the base of more holistic epidemiological studies, especially with the emergence of high-throughput molecular methods, such as NGS.

The water is a complex and readily available sample matrix for the surveillance of pathogens in the environment. Nevertheless, the rapid development of molecular techniques, namely DNA/RNA based, provide effective methods for detecting pathogens. Moreover, pathogens can have a large life expectancy in water or remnants of past infection can be detected (antigens, toxins and already non-infective viruses), and thus their detection in water being quite useful for early detections and for disease monitoring programmes.

Regarding the helminths, they have free-living phases in the soil with infecting stages inhabiting the gastrointestinal tract of hosts (faeces), thus using this type of samples can be useful

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2.3.2. Collection methods

Most of the analysed assays were based in grab-sample method, since it is the most simple and ubiquitous. This method is based on the simple collection of the environmental sample into a sterile container. Nevertheless, it requires higher sample quantity, large sample containers, people to collect the samples, higher storage capacity, among others. The eDNA techniques have been extensively used in water samples and allowing to detect multiple pathogens but also the animal hosts, namely vertebrate and invertebrate, and thus giving a relevant information on the biodiversity. Thus, water is considered the most relevant sample matrix for pathogen surveillance in the environment.

The sampling using swabs looks to be increasing, and is demonstrating that it requires lower quantity, less storage capacity and handling, and can be used in surfaces, and it can be embedded with substances for sample conservation. Nevertheless, it needs people to collect the samples and specific sterile material, which can be more expensive than simpler sterile containers as used in grab samples, as well as it not possible to automatized.

Despite not being still widely used, air sampling can be done using effective automatic sampling (automatic pumps and filters/membranes, or newly developed variations). However, these may be costly and ineffective for the surveillance of pathogens of higher mass that fall onto surfaces. Nevertheless, it can be used for detecting air transmission pathogens, namely viruses and some bacteria. The use of airborne methods is increasing, namely due to its capacity of the sampling being automatic, and continuous. Moreover, the automatic sampling may allow assessing larger areas in a lesser amount of time.

2.3.3. Pre-analytical treatment

In general, and independent of sample matrix, pre-analytic treatments are used before pathogen detections. Pathogen isolation, whether in cells, eggs, or agar, helps to remove inhibitors for more complex molecular analysis, being more often employed for the characterization of samples for the detection of the pathogen. Performed mainly for viruses (and bacteria), can also improve the assessment of the level of infection.

The sample storage conditions are an important step in pathogen detection in the environment. Storage conditions are dependent on how long the samples will remain stored prior to analysis. The ideal scenario would be the immediate analysis of the sample, especially for molecular methods, nevertheless when not possible the immediate analysis, samples are maintained refreshed and/or frozen at -20 or even at -80ºC, when for longer periods prior to analysis. This is mainly mandatory when analysing viruses and bacteria. The freeze-thawing technique helps in the recovery of genetic material from oocysts/eggs from protozoa

The ultrafiltration has been recognized as an effective procedure for concentration and recovering microbes from large volumes of water and treated wastewater.

The spiking/Internal control is very important to validate the data. The Internal Amplification Control (IAC) is a DNA sequence that is added to the PCR reaction system and is not homologous to the target gene but can be simultaneously amplified. When performing DNA extraction, it is often advantageous to have an exogenous source of DNA template that is spiked into the lysis buffer. This control DNA is then co-purified with the sample DNA and can be detected as a positive control for the extraction process.

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Moreover, method optimization, namely removing inhibitors, is very important to improve the detection success and its efficiency, especially in the DNA/RNA-based methods.

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2.3.4. Pathogen detection, discrimination, and identification

We have divided the pathogen detection and identification methods in: conventional (culture and biochemistry-based, and Immunology-based); molecular methods (nucleic acid-based methods); biosensor-based (new) and others.

Conventional methods

Culture and Biochemistry-based methods tend to be more time consuming and sometimes inconclusive, with multiple stages of culture and testing being needed for a confirmation. Give less taxonomical depth. Used as preliminary testing for other identification methods (molecular). Usually the gold-standard.

The immunology-based methods require less time to prepare the assay than a culturing technique. However, real-time pathogen detection is not possible with this method.

Many microorganisms tend to enter starvation mode of metabolism under stress conditions. However, they will remain viable but non culturable (VBNC) which cannot be grown on conventional culture (CC) media. Since no colonies will be formed, other methods such as fluorescent dyes are used for the detection of VBNC bacteria where different dyes are used.

Generally, before directly going into polymerase chain reaction (PCR) based methods, immunoassays are performed. HA is the more commonly used method in this case.

The immunology-based methods, along the general lower specificity, one other factor that affects the assay is specificity of antibody. However, since polyclonal antibodies have polyvalency (multiple epitopes to react with), it can be used for a preliminary pathogen trial detection. Nevertheless, their use can affect the reaction, leading to low specificity and sensitivity. It must be noted that there are chances of false positive results.

To overcome high detection limits, enrichment steps become important for the detection of pathogens in food products. In the enrichment step, a label-free immunoassay is used that helps in detecting the presence of the pathogen in a much simpler way. A simple and rapid detection is possible through this method with simultaneous enrichment and optical detection. The principle of this method is culture/capture/measure.

Molecular methods

The molecular methods, namely the nucleic-acid based methods are the ones more commonly and widely used for pathogen detection in environmental samples. There are several specific methods and can be used as target or multiple pathogen detection.

The nucleic-acid based methods can be developed for virtually any organism given a sufficient effort to identify specific sequences unique to the target organism.

There are several conventional PCR techniques, which are simpler, not expensive, and well established.

However, the real-time and/or quantitative PCR has been proven to be more time effective with very good specificity. Thus, these methods are by far the most used for detecting pathogens in non-invasive environmental samples

Moreover, the nucleic-acid-based techniques can provide the accurate assessment of potential hosts, which can be used simultaneously when detecting the target or multiple pathogens.

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Nevertheless, environmental samples possess a lot of possible inhibitors, which can limit the nucleic-acid-based techniques, but there are methods for removing the inhibitors. Moreover, the use of a positive control can help in the detection of false negatives and assess the role of possible inhibition in the reaction.

Biosensor-based methods

Biosensor-based methods have been developed. As example, the B1-LF-RPA, DNA-AuNP probe assay and the Radiometric colorimetric and AgNPs-fluorescence dual mode sensing for DPA based on Eu3+ have been used in pathogen detection.

In recent years, great advances have been made in nanomaterial-based biosensors, where the sensing electrode is modified by a nanomaterial to achieve a quick electron transfer due to the stimulation of different biomarkers. Due to this advantage, research has been reported where the nanomaterials are coupled with biomolecules to develop nanomaterial-based biosensors to detect dangerous pathogens. *Salmonella*, *E. coli* and *L. monocytogenes* are the most studied pathogens, where these methods have been used for their detection in food. However, the respiratory syncytial virus and parasites (*Giardia* and *Cryptosporidium*) are other pathogens that have been also detected using biosensor-based methods.

Recent modern techniques have increased sensitivity, selectivity, and stability, and allows a low detection limit for in situ measurement. The work done by Qiu et al. (2022), is a relevant demonstration on the potential of biosensor-based methods.

Others

Regarding the other methods, flow cytometry is a sensitive analytical technique which can rapidly monitor physical states of bacteria.

2.3.5. Pathogen characterization (epidemiology)

Pathogen isolation, and posterior genetic characterization has been shown to be crucial to understand pathogen evolution. Although the quantity and the quality of pathogens are usually lower in non-invasive environmental than in invasive samples, the recent development of detection, isolation and sequencing techniques allowed the assessment of pathogen characterization. The high throughput sequencing techniques allowed the assessment of degraded DNA/RNA samples, and thus potentiate the use of nucleic-acid-based techniques associated with genome sequencing.

2.4. Future prospects

The increasing use of nucleic-acid based techniques has proven the potential application and usefulness in the detection of pathogens in the environment. Several protocols have been adapted, optimized, and developed, but further research should be done, namely in natural areas, where humans, domestic animals and wildlife coexist, and relevant information on the host community exists. Moreover, these methods might allow the detection of multiple pathogens, but also to detect the potential hosts. This integrative pathogen surveillance in environmental samples needs to be better explored (e.g., The European Observatory of Wildlife). Water analysis in farms and natural ponds, which may be used simultaneously by domestic and wild animals is a great example where these studies should be performed. Case studies, such as the detection of Hepatitis A virus in environmental samples (water, soil, surface, and air) should be tested,

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including the detection of multiple hosts. Sampling, storing of different environmental samples, as well as the optimization of new techniques should be investigated to improve the wider use of these non-invasive detection methods. Moreover, biosensor-based methods have been recently used, and should be better explored, since they allow the accurate detection of pathogens, without the need of DNA/RNA extraction and amplification methods.

3. Recommendations

The main **RECOMMENDATIONS** for further implementing surveillance of zoonotic pathogens in the environment are:

- The use of environmental techniques to detect pathogens in SPs has mainly been applied to evaluate control and eradication strategies and detecting trends of zoonosis. Proven their sensitivity to detect pathogens, we also recommend the use of these techniques to detect new pathogens or unusual epidemiological events.
- Only a short proportion of SPs collects environmental samples to detect pathogens and are restricted only to some countries; and the detection and surveillance of pathogens in the environment remain untested in most for the listed pathogens of higher epidemiological importance. We recommend incorporating environmental techniques to current SPs focusing on zoonotic prioritized pathogens since this has been done in only a minority of the SPs. Results must be compared with conventional surveillance in such ongoing SPs to evaluate their sensitivity and cost/effectiveness, especially for early warning/detection of zoonotic pathogens.
- The integration and use of environmental pathogen detection by different SPs and health sectors is recommended to address multi-pathogen multi-host disease surveillance. This approach can benefit from the combined use of different types of environmental samples to detect pathogens (such as water and biological materials, to a lesser extent soil, and other matrices), and should include vectors as matrices where pathogens can also be detected.
- The use of environmental techniques to detect pathogens is highly recommended for natural habitats where most host reservoirs of the listed priority zoonosis are present, targeting the appropriate sample matrix, such as water-based samples.
- Nucleic acid-based methods can be developed for virtually any organism, given a sufficient effort to identify specific DNA/RNA sequences unique to the target organism, and can also provide accurate assessment of potential hosts, which can be used simultaneously when detecting the target or multiple pathogens. It is recommended that this approach is better explored and tested to perform integrative pathogen surveillance in environmental samples (e.g., the European Observatory of Wildlife).
- Water analysis in farms and natural ponds, which may be used simultaneously by domestic and wild animals, is a good example where new research should be conducted. Case studies using environmental samples (water, soil, surface, and air) should be promoted, including the detection of multiple hosts using metagenomic approaches.
- Finally, the use of biosensor-based methods has increased in the last years, they are very promising and should be better explored since they allow the accurate detection of pathogens, without the need of DNA/RNA extraction and amplification methods.

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- For the literature review on the main existing structures and systematic, and academic initiatives for surveillance in the EU for zoonoses in the environment See complete list of references reviewed in Annex 1 (https://doi.org/10.5281/zenodo.7446382).
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Supplementary Tables

Supplementary Table 1. List of pathogen identification methods recorded in the analyzed assays. Given is the full name and respective abbreviation, when called for, of the method variants from each defined method category.

Abbreviation	Name
Nucleic acid-based Methods	
B1-LF-RPA	Recombinase Polymerase Amplification of the B1 gene visualized by a Lateral Flow strip
Conventional PCR	Conventional Polymerase Chain Reaction
	CRISPR/Cas12a
ddPCR	drop digital PCR
DNA-AuNP probe assay	Deoxyribonucleic acid-Gold NanoParticle probe assay
ICC-qPCR	Integrated Cell Culture quantitative PCR;
IR-NAAS	Interference Reduction Nucleic Acid Amplification Strategy
LAMP	Loop-mediated isothermal Amplification
MFqPCR	Microfluidic quantitative PCR
MRT-PCR	Multpilex Real-Time PCR
Multiplex qPCR	Multiplex quantitative-PCR
Nested RT-PCR	Nested Real-Time PCR
NGS	Next Generation Sequencing
PCR-RFLP	Restriction Fragment Length Polymorphism PCR
qPCR	quantitative-PCR
rRT-PCR	Real-Time reverse transcription PCR
rRT-qPCR	Real-Time quantitative reverse transcription PCR
RT-ddPCR	Real-Time drop digital PCR
RT-LAMP	Real-Time LAMP
RT-PCR	Real-Time PCR
RT-qPCR	Real-Time quantitative PCR
RV-PCR	Rapid Viability PCR
RV-RT-PCR	Rapid Viability Real-Time PCR
Sanger sequencing	
SNP-RT-PCR	Single Nuclear Polymorphisms Real-Time PCR
Immunology-based Methods	
DFA	Direct Immunofluorescence Assay;
EIA	Enzime Immunoassay
ELISA	Enzyme-Linked Immunosorbent Assay
HA	Hemagglutination test
HI	Hemagglutination Innhibition test

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IFA	Immunofluorescence Assay
IFAT	Quantitative Immunofluorescence Assay
IMS-IFA	Immunomagnetic Separation – IFA
Latex Agglutination Test	
MAT	Microscopic Agglutination Test;
Culture and Biochemistry-based Methods	
	API 20E Strip test
	Biotyping
	Chromogenic Substract technique
	Colilert test
	Count Plates
	Cytochrome c Oxidase test
	Darkfield microscopy
	Epifluorescence Microscopy
	Flotation technique
	Fluorescence Microscopy
	Gram staining
	Hippurate Hydrolysis test
	Light Microscopy
	Microbiologic Culture
	MicroScan system
MPN	Most Probable Number technique
	Neubauer technique
	Paper-based Eletrochemical Quantification
	Slide Agglutination test
	Ziehl-Neelsen staining
Other	
	Flow cytometry
	Fluorometry
	Radiometric colorimetric and AgNPs-fluorescence dual mode sensing
MALDI-TOF-MS	Matrix-Assisted Laser Desorption/Ionization-Time Of Flight-Mass Spectrometry

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Supplementary Table 2. Description of Bacteria identification methods based on the anlaysed publications. Given is the method variant, pre-analytical treatment strategies, type of sample matrix and target hazard, by method category (Nucleic acid-based; Immunology-based; Culture and Biochemistry-based; Others). In the Pre-treatment column are pre-analytical treatments performed in one or more defined assays, but not necessarily within the same assay. References using the described methods are shown.

Nucleic acid-based methods					
#	**Method**	**Pre-treatment**	**Sample Type (matrix)**	**Target hazard**	**References**
1.	Conventional PCR	Sample concentration Pathogen isolation DNA extraction	Faecal Object surface Soil Other (Urine) Water Other (Feed)	*Coxiella burnetii* *Leptospira spp.* Other	Byeon et al., 2022 Pande et al., 2020 Dhaka et al., 2019 Nuthong et al., 2018 Yap et al., 2021 Yap et al., 2019 binti Daud et al., 2018
2.	DNA-AuNP probe assay	DNA extraction	Water	Other	Nuthong et al., 2018
3.	Multiplex PCR	DNA extraction	Water	*Leptospira spp.*	Ospina-Pinto et al., 2021
4.	Multiplex qPCR	Sample concentration DNA extraction	Water Object surface	*Leptospira spp.* *Francisella tularensis* *Coxiella burnetii*	Richard et al., 2022 Janse et al., 2018 Carrié et al., 2019
5.	Nested PCR	DNA extraction	Soil	Other	Soto et al., 2017
6.	qPCR	Sample concentration DNA extraction	Water Soil Object surface	*Leptospira spp.* *Francisella tularensis* Other	Wilkinson et al., 2021 Brunet et al., 2021 Sevillano et al., 2021 Fuhrmeister et al., 2019 Huvarova et al., 2018 Gonzales-Gustavson et al., 2017 Beigel & Verma, 2017

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7.	RT-PCR	Saple concentration Freeze-Thaw technique DNA extraction	Object surface Wastewater Water Soil	*Brucella (B. abortus, melitensis, suis)* *Coxiella burnetii* *Francisella tularensis* *Leptospira spp.* Other	Rebollada-Merino et al., 2022 Sadeghi et al., 2022 Liu et al., 2021 Zendoia et al., 2021 Cohan et al., 2020 Cortez et al., 2018 Cieślik et al., 2018 Hurtado et al., 2017 Ahmed et al., 2017
8.	RT-qPCR	Sample concentration DNA extraction	Object surface Water	Other	Dziedzinska et al., 2018
9.	RV-PCR	DNA extraction	Water	*Francisella tularensis*	Kane et al., 2019
10.	Sanger sequencing (Conventional PCR/Nested PCR/RT-PCR)	Sample concentration DNA extraction	Faecal Soil Water	*Leptospira spp.* Other	Kumari et al., 2021 Chaiwattanarungruengpaisan et al., 2020 Zulkifli et al., 2018 Ali et al., 2018 Pui et al., 2017 Soto et al., 2017
11.	SNP-Rti-PCR	Sample concentration DNA extraction	Object surface	*Coxiella burnetii*	Zendoia et al., 2021
Immunology-based methods					
#	**Method**	**Pre-treatment**	**Sample Type (matrix)**	**Target hazard**	**References**
1.	Latex agglutination test		Water	Other	Nuthong et al., 2018
2.	MAT		Water Other (Urine)	*Leptospira spp.*	Ospina-Pinto et al., 2021
Culture and Biochemistry-based methods					
#	**Method**	**Pre-treatment**	**Sample Type (matrix)**	**Target hazard**	**References**
1.	API 20E Strip test	Pre-enrichment	Sewage	Other	Flemming et al., 2017
2.	Biotyping	Pre-enrichment	Faecal Sewage	Other	Rai et al., 2019

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3.	Colilert test	Sample concentration	Water Soil Object surface	Other	Souza et al., 2020 Rai et al., 2019 Fuhrmeister et al., 2019
4.	cytochrome C oxidase test	Sample concentration	Faecal	Other	Contreras et al., 2017
5.	Dark-field microscopy	Sample concentration Pre-enrichment	Water Sewage Soil Other (Urine) Other (Feed)	*Leptospira spp.* Other	Baki et al., 2020 Zaki et al., 2020 Narkkul et al., 2020 Chaiwattanarungruengpaisan et al., 2020 Pande et al., 2020 Flemming et al., 2017
6.	Gram staining test	Sample concentration	Faecal	Other	Contreras et al., 2017
7.	Hippurate Hydrolysis test	Sample concentration	Faecal	Other	Contreras et al., 2017
8.	Microbiologic culture	Sample concentration Pre-enrichment Selective enrichment	Soil Water Object surface Faecal Wastewater Sewage	*Bacillus anthracis* *Leptospira spp.* Other	Rohde et al., 2020 Bailey et al., 2020 Barandongo et al., 2018 Dziedzinska et al., 2018 Cortez et al., 2018 Huvarova et al., 2018 Contreras et al., 2017 Flemming et al., 2017
9.	MicroScan system	Sample concentration	Faecal	Other	Contreras et al., 2017
10.	MPN		Water Object surface Sewage	Other	Bailey et al., 2020 Contreras et al., 2017 Flemming et al., 2017
11.	Paper-based Eletrochemical Quantification		Air	*Bacillus anthracis*	Park et al., 2022
12.	Slide agglutination	Pre-enrichment	Sewage	Other	Flemming et al., 2017
Other methods					

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#	Method	Pre-treatment	Sample Type (matrix)	Target hazard	References
1.	Radiometric colorimetric and AgNPs-fluorescence dual mode sensing	Sample concentration	Water	*Bacillus anthracis*	Qiu et al., 2022 Yin & Tong, 2021

Supplementary Table 3. Description of Virus identification methods based on the analysed publications. Given is the method variant, pre-analytical treatment strategies, type of sample matrix and target hazard, by method category (Nucleic acid-based; Immunology-based; Culture and Biochemistry-based; Others). In the Pre-treatment column are pre-analytical treatments performed in one or more defined assays, but not necessarily within the same assay. References using the described methods are shown.

Nucleic acid-based methods					
#	Method	Pre-treatment	Sample Type (matrix)	Target hazard	References
1.	Conventional PCR	Sample concentration RNA extraction cDNA synthesis	Wastewater Object surface	SARS-Coronavirus type 2 Influenza A virus (Avian)	Bar-Or et al., 2021 Henning et al., 2019
2.	ICC-qPCR	Sample concentration	Water	Ad helper virus	Bailey et al., 2020
3.	IR-NAAS	mbRCA-based amplification	Sewage	SARS-Coronavirus type 2	Chen et al., 2022
4.	MFqPCR	Sample concentration RNA extraction cDNA synthesis	Wastewater	Hepatitis E virus	Kobayashi et al., 2017
5.	Multiplex RT-PCR	RNA extraction cDNA synthesis	Object surface Air	Influenza A virus (Avian)	Ikonen et al., 2018 Crank et al., 2020 Iaconelli et al., 2020
6.	Nested RT-PCR	RNA extraction cDNA synthesis	Water Wastewater Sewage	Hepatitis E virus	Souza et al., 2020
7.	NGS (HiSeq/MiSeq/NextSeq)	Sample concentration RNA extraction DNA degradation	Wastewater Faecal Water Soil	Hepatitis E virus Other	Adriaenssens et al., 2018 Oshiki et al., 2018 Ramírez et al., 2020

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		RNA concentration			
8.	qPCR	Sample concentration Pathogen isolation RNA extraction cDNA synthesis	Wastewater Water Object surface Air Soil	SARS-Coronavirus type 2 Hepatitis E virus Other	Izzotti et al., 2022 Souza et al., 2020 Hong et al., 2021 Zahedi et al., 2020 Fuhrmeister et al., 2019
9.	rRT-PCR	Sample concentration Pathogen isolation RNA extraction	Object surface Soil Air Water Faecal Other (Cloacal/Anal sample) Other (Biofilm)	SARS-Coronavirus type 2 Influenza A virus (Avian) Influenza A virus (Swine) Hepatitis E virus	Cooper et al., 2018 Gonzales-Gustavson et al., 2017 Huneau-Salaün et al., 2022 Storms et al., 2021 Huneau-Salaün et al., 2020 Germeraad et al., 2020 Ramírez et al., 2020 Nolting et al., 2020 Garrido-Mantilla et al., 2019 Wu et al., 2019 Khan et al., 2018 Muñoz-Aguayo et al., 2019 Sayeed et al., 2017 Azeem et al., 2021 Germeraad et al., 2020 Muñoz-Aguayo et al., 2019 Poulson et al., 2017 Filaire et al., 2022 Garrido-Mantilla et al., 2020

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10.	RT-ddPCR	Sample concentration RNA extraction	Wastewater Air Water Object surface	SARS-Coronavirus type 2	Conte et al., 2022 Grijalva et al., ? Kim et al., 2022 Cardinale et al., 2022 Flood et al., 2021
11.	RT-LAMP	DNA/RNA extraction	Faecal	Influenza A virus (Avian)	Onuma et al., 2017
12.	RT-PCR	Sample concentration Pathogen isolation RNA extraction cDNA synthesis	Wastewater Air Object surface Faecal Water Sewage Other (Cloacal/Anal sample)	SARS-Coronavirus type 2 Influenza A virus (Avian) Influenza A virus (Swine) Hepatitis E virus Other	Conte et al., 2022 Bar-Or et al., 2021 Zhang et al., 2021 Lopez Moreno et al., 2021 Ben-Shmuel et al., 2020 Cheng et al., 2020 Dharmayanti et al., 2020 Lau et al., 2019 Yang et al., 2019 Markantonis et al., 2018 Kim et al., 2018 Wang et al., 2018 Tun Win et al., 2017 Welling et al., 2022 Cardinale et al., 2022 Liu et al. 2021 Bo et al., 2021 Serra-Compte et al., 2021 Barril et al., 2021 Fongaro et al., 2021 Yeager et al., 2021 Peng et al., 2018 Caruso et al., 2017 Pawar et al., 2021 Wong et al., 2021 Souza et al., 2020

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13.1	RT-qPCR	Sample concentration RNA extraction RNA concentration cDNA synthesis	Wastewater Sewage Air Water Soil Other (Does not specify)	SARS-Coronavirus type 2 Influenza A virus (Avian) Hepatitis E virus	Oh et al., 2022 El-Malah et al., 2022 Stobnicka-Kupiec et al., 2022 Bayati et al., 2022 Kim et al., 2022 Alamin et al., 2022 Hewitt et al., 2022 Deng et al., 2022 Amman et al., 2022 Mondal et al., 2021 Chik et al., 2021 Farkas et al., 2021 Bar-Or et al., 2021 Serra-Compte et al., 2021 La Rosa et al., 2021 Wang et al., 2021 Flood et al., 2021 D'Aoust et al., 2021 Salvador et al., 2020 Beyer et al., 2020 Wei et al., 2018 Lickfett et al., 2018 Schaeffer et at., 2018 Dziedzinska et al., 2018 Anderson et al., 2018 Cooper et al., 2018 Huvarova et al., 2018 Adriaenssens et al., 2018 Zhu et al., 2022 Pino et al., 2021 D'Aoust et al., 2021 Masachessi et al., 2018 Bailey et al., 2021

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#	Method	Pre-treatment	Sample Type (matrix)	Target hazard	References
13.2	RT-qPCR	Sample concentration Dye treatment DNA/RNA extraction	Air Object surface Wastewater	SARS-Coronavirus type 2 Influenza A virus (Avian)	Stobnicka-Kupiec et al., 2022
13.3	RT-qPCR	Sample concentration Plasmid cloning DNA/RNA extraction	Object surface	Hepatitis E virus	Di Profio et al., 2019
14.	RV-RT-PCR	Sample concentration RNA extraction	Object surface	SARS-Coronavirus type 2	Shah et al., 2021
15.	Sanger sequencing (Nested PCR)	Sample concentration RNA extraction cDNA synthesis	Water	Hepatitis E virus	La Rosa et al., 2018 Pisano et al., 2018
			Immunology-based methods		
#	**Method**	**Pre-treatment**	**Sample Type (matrix)**	**Target hazard**	**References**
1.	DFA	Sample concentration Sample clarification	Water	Other	Masachessi et al., 2018
2.	EIA	Sample concentration	Faecal	Other	Contreras et al., 2017
3.	HA	Pathogen isolation	Object surface Faecal Water Other (Cloacal/Anal sample)	Influenza A virus (Avian)	Chen et al., 2019 Muzyka et al., 2019 Biswas et al., 2018 Khan et al., 2018
4.	HI	Pathogen isolation	Sewage Faecal Water Other (Cloacal/Anal sample)	Influenza A virus (Avian)	Chen et al., 2019 Muzyka et al., 2019
			Culture and Biochemistry-based methods		
#	**Method**	**Pre-treatment**	**Sample Type (matrix)**	**Target hazard**	**References**

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#	Method	Pre-treatment	Sample Type (matrix)	Target hazard	References
1.	Count plates	Incubation	Object surface	SARS-Coronavirus type 2	Huneau-Salaün et al., 2022
2.	Microbiologic culture		Air Object surface Soil Other (Leaves)	Influenza A virus (Swine) Hepatitis E virus	Qiao et al., 2021 Mikelonis et al., 2020
Other methods					
#	**Method**	**Pre-treatment**	**Sample Type (matrix)**	**Target hazard**	**References**
1.	Fluorimetry		Air	Influenza A virus (Swine) Hepatitis E virus	Qiao et al., 2021

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Supplementary Table 4. Description of Protozoa identification methods based on the analysed publications. Given is the method variant, pre-analytical treatment strategies, type of sample matrix and target hazard, by method category (Nucleic acid-based; Immunology-based; Culture and Biochemistry-based; Others). In the Pre-treatment column are pre-analytical treatments performed in one or more defined assays, but not necessarily within the same assay. References using the described methods are shown.

Nucleic acid-based methods					
#	**Method**	**Pre-treatment**	**Sample Type (matrix)**	**Target hazard**	**References**
1.	B1-LF-RPA assay	Sample concentration Freeze-Thaw technique DNA extraction	Soil Water	*Toxoplasma gondii*	Wu et al., 2017
2.	Conventional PCR	Sample concentration Freez-Thaw technique DNA extraction	Soil Faecal Water Other (Feed)	*Cryptosporidium spp.* *Toxoplasma gondii* *Leishmania spp.*	Bernardes et al., 2021 Al-Warid et al., 2019 Davis et al., 2018 Soto et al., 2017
3.	CRISPR/Cas 12a		Soil	*Cryptosporidium spp.* *Toxoplasma gondii*	Li et al., 2021 Ma et al., 2021
4.	ddPCR	DNA extraction	Wastewater	*Cryptosporidium spp.*	Mthethwa et al., 2022
5.	LAMP	Sample concentration Wash Freeze- Thaw technique DNA extraction	Faecal Water Other (Vegetable)	*Giardia spp.*	Lalonde et al., 2021 Solarczyk et al., 2019 Lass et al., 2017
6.	Multiplex RT-PCR	DNA extraction	Sewage	*Cryptosporidium spp.* *Giardia spp.*	Vassalosa et al., 2017

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7.	Nested PCR	Sample concentration Wash Freeze- Thaw technique DNA extraction	Water Sewage Soil Faecal Other (Cloacal/Anal sample) Other (Vegetable)	*Cryptosporidium spp.* *Giardia spp.* *Leishmania spp.* *Toxoplasma gondii*	Hublin et al., 2022 Mphephu et al., 202 Vejano et al., 2021 Lalonde et al., 2021 Martins et al., 2019 Vassalosa et al., 2017 Lass et al., 2017 Soto et al., 2017 Wu et al., 2017
8.	NGS (MiSeq)	Sample concentration DNA extraction	Wastewater	*Cryptosporidium spp.*	Zahedi et al., 2019
9.	PCR-RFLP	DNA extraction	Faecal	*Cryptosporidium spp.*	Ng-Hublin et al., 2017
10.	qPCR	Sample concentration DNA extraction	Water Soil Faecal Wastewater Soil Object surface Other (Cloacal/Anal sample)	*Cryptosporidium spp.* *Giardia spp.* Other	Mphephu et al., 2021 Medkour et al., 2020 Zahedi et al., 2019 Fuhrmeister et al., 2019 Zahedi et al., 2018 Dziedzinska et al., 2018 Moreno et al., 2018 Zahedi et al., 2018 Huvarova et al., 2018 Squire et al., 2017
11.	RT-PCR	Sample concentration Freeze- Thaw technique DNA extraction	Wastewater Faecal Water	*Cryptosporidium spp.* *Giardia spp.* Other	Sadeghi et al., 2022 Liu et al., 2021 Braima et al., 2021 Menu et al., 2021 de Souza et al., 2019 Lass et al., 2017

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12.	Sanger sequencing (Conventional PCR/Nested PCR)	Sample concentration Freeze- Thaw technique DNA extraction	Faecal Water Wastewater	*Leptospira spp.* *Giardia spp.* *Toxoplasma gondii*	Kumari et al., 2021 Masangkay et al., 2020 Li et al., 2020 Zahedi et al., 2019 Braima et al., 2019 Zahedi et al., 2017
Immunology-based methods					
#	**Method**	**Pre-treatment**	**Sample Type (matrix)**	**Target hazard**	**References**
1.	DFA	Sample concentration	Faecal Sewage	*Cryptosporidium spp.* *Giardia spp.* Other	Smith et al., 2020 Vassalosa et al., 2017
2.	ELISA	Sample concentration	Faecal	*Cryptosporidium spp.* *Giardia spp.*	Contreras et al., 2017
3.	Ether-IMS-IFA	Sample concentration	Faecal	*Cryptosporidium spp.* *Giardia spp.*	Li et al., 2020
4.	IFA	Sample concentration	Faecal Wastewater Water	*Cryptosporidium spp.* *Giardia spp.* Other	Li et al., 2020 de Souza et al., 2019 Moreno et al., 2018) Gonzales-Gustavson et al., 2017 Contreras et al., 2017 Hatam-Nahavandi et al., 2017
5.	IFAT	Sample concentration	Wastewater	*Cryptosporidium spp.* *Giardia spp.*	Ligda et al., 2020
6.	IMS	Sample concentration	Wastewater	*Cryptosporidium spp.* *Giardia spp.*	Ligda et al., 2020
7.	IMS-IFA	Sample concentration	Faecal	*Cryptosporidium spp.* *Giardia spp.*	Li et al., 2020

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8.	NaPP-IMS-IFA	Sample concentration	Faecal	*Cryptosporidium spp.* *Giardia spp.*	Li et al., 2020
Culture and Biochemistry-based methods					
#	**Method**	**Pre-treatment**	**Sample Type (matrix)**	**Target hazard**	**References**
1.	Chromogenic substrate technique		Water	*Toxoplasma gondii*	Bernardes et al., 2021
2.	Epifluorescence microscopy	Sample concentration	Wastewater Sewage Water Soil Other (Vegetable)	*Cryptosporidium spp.* *Giardia spp.*	Tram et al., 2022 Bailey et al., 2020
3.1	Fluorescence microscopy (HPF)	Sample concentration Auramine (Aura) fluorescent technique	Water Other (Biofilm)	*Cryptosporidium spp.*	Masangkay et al., 2020
3.2	Fluorescence microscopy (HPF)	Sample concentration Direct Antibody Fluorescence Technique (IFT)	Water Other (Biofilm)	*Cryptosporidium spp.*	Masangkay et al., 2020
4.1	Light microscopy (OIF)	Sample concentration Modified Kinyoun's (MK) stain	Water Other (Biofilm)	*Cryptosporidium spp.*	Masangkay et al., 2020
4.2	Light microscopy (OIF)	Sample concentration Modified Safranin Methylene Blue (SMB) stain	Water Other (Biofilm)	*Cryptosporidium spp.*	Masangkay et al., 2020
5.	MPN		Sewage	*Cryptosporidium spp.* *Giardia spp.*	Flemming et al., 2017
6.	Neubauer technique	Sample concentration	Faecal	*Cryptosporidium spp.*	Gathercole et al., 2021

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#	Method	Pre-treatment	Sample Type (matrix)	Target hazard	References
7.	Ziehl-Neelsen staining	Sample concentration	Water Soil Faecal	*Cryptosporidium spp.*	Mphephu et al., 2021 Braima et al., 2021
			Other methods		
#	**Method**	**Pre-treatment**	**Sample Type (matrix)**	**Target hazard**	**References**
1.	Flow cytometry	Sample concentration	Wastewater Water	*Cryptosporidium spp.* *Giardia spp.*	Hassan et al., 2021 Göröcs et al., 2020
2.	MALDI-TOF	Sample concentration	Faecal	*Cryptosporidium spp.*	Gathercole et al., 2021

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Supplementary Table 5. Description of Helminth identification methods based on the analysed publications. Given is the method variant, pre-analytical treatment strategies, type of sample matrix and target hazard, by method category (Nucleic acid-based; Immunology-based; Culture and Biochemistry-based; Others). In the Pre-treatment column are pre-analytical treatments performed in one or more defined assays, but not necessarily within the same assay. References using the described methods are shown.

Nucleic acid-based methods					
#	**Method**	**Pre-treatment**	**Sample Type (matrix)**	**Target hazard**	**References**
1.	LAMP	DNA extraction	Faecal	*Echinococcus multilocularis* Other	Avila et al., 2020
2.	Multiplex qPCR	Sample concentration DNA extraction	Soil Faecal	*Echinococcus granulosus* Other	Umhang et al., 2017
3.	qPCR	DNA extraction	Faecal	Other	Medkour et al., 2020
4.	RT-PCR	Sample concentration DNA extraction	Soil Faecal	*Echinococcus multilocularis*	Da Silva et al., ?
5.	RT-qPCR	DNA extraction	Soil	Other	Jarosz et al., 2021
Culture and Biochemistry-based methods					
#	**Method**	**Pre-treatment**	**Sample Type (matrix)**	**Target hazard**	**References**
1.	Flotation technique (Sheather method/Teleman n method)		Faecal	*Echinococcus granulosus*	Avila et al., 2020
2.	Light microscopy	Sample concentration	Faecal Sewage	Other	Rai et al., 2019 Zdybel et al., 2019

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