Monitoring oomycetes in water: combinations of methodologies used to answer key monitoring questions

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Monitoring oomycete populations and communities in bodies of water is vital in developing our understanding of this important group of fungus-like protists that contains many serious pathogens of both crops and wild plants. The methodologies involved in monitoring oomycetes are often presented as a developmental hierarchy, progressing from 'traditional' culture-based techniques through immunological techniques and basic PCR to qPCR and metagenomics. Here, techniques are assessed according to the roles they can perform in relation to four stages of the monitoring process: capture, detection and identification, viability determination, and quantification. Possible synergies are then considered for the combined use of different techniques in addressing the various needs relating to different questions asked of monitoring, with an emphasis on the continuing value of cultural and immunodiagnostic procedures. Additionally, the exciting future presented by the ongoing development and improvement of metabarcoding and the use of high throughput sequencing techniques in the measurement and monitoring of oomycete inoculum to determine and mitigate plant disease risks is addressed.

KEYWORDS

oomycetes, water, detection, propagule-viability, quantification, monitoring

1 Introduction

The Oomycetes are a diverse group of filamentous protists in the clade *Stramenopiles.* As indicated by their old name, 'the water molds', oomycetes generally thrive in aquatic environments, with free water vital for their dispersal and supporting a major part of their asexual lifecycles (Hüberli et al., 2013). Many members of the oomycetes are saprotrophic, but a significant proportion of species are also endophytic and/or parasitic. It is claimed that oomycetes are likely all 'hard wired' for parasitism (Beakes et al., 2012) with many early divergent genera in their phylogenetic tree being marine parasites of a diverse range of organisms. In water used for horticulture, members of the orders Peronsporales, Pythiales, and Saprolegniales are of particular importance containing many important plant pathogen genera capable of causing devastating diseases in crops, ornamentals, and

native plants (Kamoun, 2009). *Phytophthora* and *Pythium* are probably the most notorious oomycete genera and have received the greatest attention. In horticultural practice water used for irrigation, is obtained from a wide range of sources including boreholes and wells, roof-harvested rainwater, water from rivers, lakes and ponds as well as re-cycled hydroponics feed solutions and run-off or tail-water retrieved from production beds (Moorman et al., 2014). The potential risks of contamination of such water sources with plant pathogen inoculum can vary enormously (Moorman et al., 2014) and it is important to monitor water entering and leaving nurseries and gardens for oomycetes to guide management and regulation of disease risks, both in terms of potential crop loss and the threats of greater damage from spread to the wider environment (Jung et al., 2016; Barwell et al., 2020; Green et al., 2021; Dale et al., 2022). The continued development and refinement of measuring and monitoring techniques for oomycetes is essential for building understanding of their lifecycles, dispersal, etiology, and potential environmental impacts. Greater understanding of these factors will help determination and mitigation of disease risks and reduce damage and economic losses. For valid determinations of disease risks, accurate identification, and quantification of inoculum in time and space and in relation to survival, infection, and disease development are vital. The primary oomycete propagules in water are likely sporangia (when caducous), zoospores, and zoospore cysts (Weste, 1983). A great deal is understood about zoospore behavior, especially in the prelude to infection (Judelson and Blanco, 2005; Bassani et al., 2020), although much of this work focusses on interactions within the soil and the rhizosphere and it has often been assumed to be similar in water. However, in water, there are extra factors that are rarely considered concerning zoospore activity in larger bodies of water, where repetitional diplanetism (Drechsler, 1930; Moralejo and Descals, 2011) and zoospore cysts may play an important role in long-distance movement and survival.

Over 400 *Phytophthora* and *Pythium* and allied species have now been identified. Only a relatively small proportion of these are readily isolated and regularly seen in water samples (65+ including species of *Phytophthora, Pythium, Phytopythium*, and *Halophytophora*: Hwang et al., 2008; Reeser et al., 2011; Hüberli et al., 2013; Huai et al., 2013; Nagel et al., 2013; Zappia et al., 2014; Choudhary et al., 2016; Stamler et al., 2016; Nam and Choi, 2019; Redekar et al., 2019; Riolo et al., 2020). However, it seems likely that many more species are in fact able to spread in bodies of water. During the process of zoospore aggregation prior to root infection, there is evidence of interspecific cooperation (Kong et al., 2010), which may explain why some widespread oomycete pathogens are less frequently detected in water but still appear in root infections. There may be some merit in comparing species mixes in inoculation experiments, which by and large only assess single introductions of single putative pathogens (Hong, 2014).

The techniques developed and used for the monitoring and measurement of oomycetes in water are essentially very similar to, or the same as those developed for other media such as growing substrates, soils or colonised plant tissues. This review refers were possible to published methods applied to test and measure water. Techniques are first outlined in relation to four proposed stages of water assessment. Their varying applicability to water testing scenarios addressing different questions is then considered with the aim of encouraging the use of multiple methods and including culturing techniques where possible.

2 Stages in the process of monitoring oomycetes in water

Effective monitoring and measurement of oomycetes in water can be broken down into four stages: 1) capture, 2) detection and identification, 3) determination of viability, and 4) quantification. Each of these stages and the techniques applicable to them are considered in more detail below and summarized in Figure 1. The capture of detectable oomycete biomass from water is considered as a separate process here because of its high importance in the monitoring of water, even though some capture techniques such as baiting in many circumstances are also effective for detection in their own right which is in contrast to capture approaches like filtration that are purely effective for the physical collection of particles from water. A number of different very sensitive approaches can be used for detection, while relatively recent developments, especially in the field of nucleotide-based chemistries, have greatly expanded the scope for identifications, quantification, and the exploration of contextual information such as wider microbiomes (for example Ruiz Gómez et al., 2019). This exploration is opening up great new opportunities for understanding pathosystems, although such methods still have problems with determinations of viability. There is always room for improvement, but the range of methods now possible means the investigator can develop and tailor an approach to monitoring oomycetes based on the nature of the measurement question(s) being asked (Figure 2).

2.1 Capture

The central challenge to successful monitoring of aquatic oomycetes is that the amount of target biomass is very small and potentially widely and unevenly dispersed amongst many other taxa and debris in large volumes of water. Effective capture of oomycete biomass from water is essential and is currently achieved largely either by baiting or by filtration methods. Other potential methods include centrifugation and magnetic antibody concentration. Centrifugation has been deployed to extract viable *Phytophthora* zoospores (most likely as zoospore cysts after centrifugation) from hardy nursery-stock irrigation water (Middleton, 1985). Unwieldy, involving the separation of larger samples into 250 ml aliquots for centrifugation and plating out resuspended pellets onto agar growing media, this method is time-consuming and not efficient for large numbers of samples. In comparison trials, centrifugation was also found to be less effective than other capture methods for molecular detection of *Phytophthora* in water samples (Scibetta et al., 2012). Immunomagnetic separation, is a process that can be either direct or indirect. Direct immunomagnetic separation

Stages in water monitoring / **Water monitoring techniques**

Stages in water monitoring	Membrane filters	Baiting	Colony Forming Units	Zoospore-Trapping Immunoassay	Dipsticks	Lateral Flow Devices	Enzyme-Linked Immuno-Sorbent Assay	PCR	qPCR	Meta-barcoding
Concentration /capture	Very good to excellent	Very good to excellent	Not effective	Very good to excellent	Good	Not effective	Not effective	Not effective	Not effective	Not effective
Detection/ identification	Not effective	Good	Good	Good	Moderately effective to good	Moderately effective to good	Moderately effective to good	Good	Good	Very good to excellent
Viability	Not effective	Very good to excellent	Very good to excellent	Very good to excellent	Very good to excellent	Not effective	Not effective	Not effective	Not effective	Not effective
Quantification	Not effective	Moderately effective to good	Very good to excellent	Very good to excellent	Good	Moderately effective to good	Very good to excellent	Not effective	Very good to excellent	Very good to excellent

KEY

○ Not effective
● (yellow) Moderately effective to good
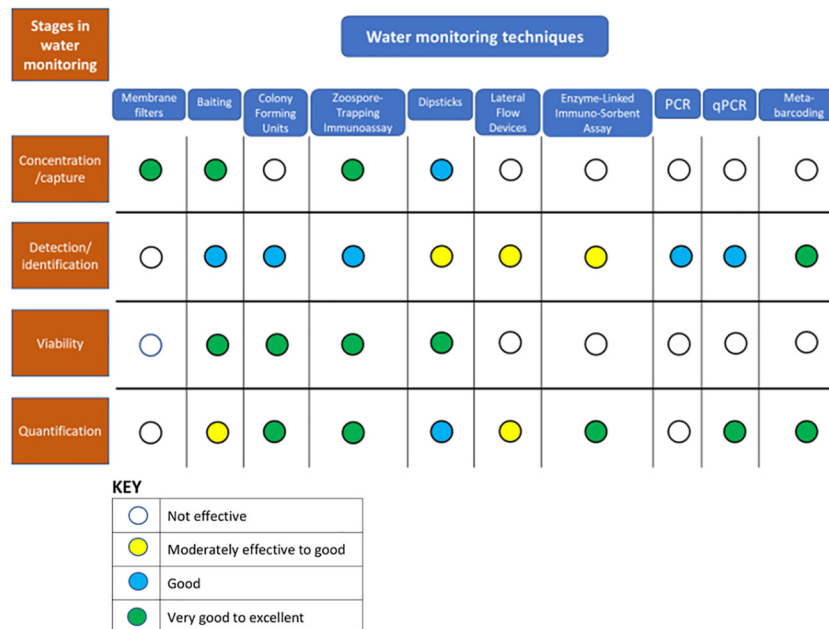● (blue) Good
● (green) Very good to excellent

FIGURE 1
Consideration of the process of monitoring oomycetes in water samples as a series of stages: capture, detection/identification, viability assessment and quantification, and the relative suitability of a range of water monitoring techniques for each of these stages.

deploys target-specific antibodies directly conjugated to super paramagnetic spheres (Cheng et al., 2010; Kennedy and Wakeham, 2013), whereas indirect deploys mouse antibodies raised to the target and then uses an anti-mouse antibody conjugated to magnetic spheres to bind to these. In both cases, the antibodies are introduced to samples where they can bind to target biomass and can then be isolated from the samples for further diagnostic tests by exposure to a magnetic field. Despite showing efficacy in the extraction of cysts of other pathogenic protists from water samples (e.g. *Cryptosporidium*, Campbell and Smith, 1997, and *Giardia lamblia*, Bifulco and Schaefer, 1993), this approach has not yet been reported for the successful capture of oomycete propagules from environmental water samples.

2.1.1 Baiting

Since the 1960s, use has been made, primarily of plant tissues, to act as baits to attract oomycete spores out of bodies of water via chemo-attraction and chemotaxis. Many different nuances of the baiting approach exist, and the earliest of these techniques used for *Phytophthora* species have been comprehensively catalogued by Ribeiro (1978). As they are low in cost, straightforward, and highly adaptable, baiting techniques are still widely developed and successfully used for the capture of oomycetes in water. The baits selected for an assay strongly influence the resulting catch: for example, autoclaved insect parts are likely to attract Saprolegniales and some *Pythium* species (Sarowar et al., 2013), whereas fresh, whole rhododendron leaves work well for *Phytophthora* species in temperate streams and hardy nursery-stock irrigation systems (Themann and Werres, 1998; Themann et al., 2002). Green et al. (2020) effectively deployed a mix of different fresh leaf species as baits in a study investigating the range of *Phytophthora* species

present in public parks, whilst in comparative trials in Australia, primarily looking at soils, a multiple bait system used by the Centre for Phytophthora Science and Management was the most effective baiting procedure (Burgess et al., 2021). The age, quality, and physiological state of the plant tissues being used as baits are hugely influential on the results obtained (Themann et al., 2002; Hüberli et al., 2013; Werres et al., 2014), and inclusion of dead tissues within a bait mix can increase the number of species captured (Wielgoss et al., 2009; Aram and Rizzo, 2018; Sarker et al., 2023a). When baiting is deployed in comparative experiments over time, variability can be reduced by using more controllable tissues such as seedlings or seedling parts (Banihashemi and Mitchell, 1975; Erwin and Ribeiro, 1996; Pettitt et al., 1998). Perhaps the ultimate in reproducible baits use just chemical attractants and do not have to rely on maintaining consistency of plant material. This principle was successfully deployed using phenols, alcohols, and amino acids in the development of chemo-attractive dipsticks to detect viable *Phytophthora cinnamomi*, and later *P. nicotianae*, zoospores (Cahill and Hardham, 1994a and Cahill and Hardham, 1994b; Gautam et al., 1999). Nevertheless, plant tissue-based baits continue to predominate and be widely and successfully deployed for the capture of oomycetes from water.

Irrespective of the materials used for baits, there are two main approaches to baiting water; *in situ* and *ex situ* (Werres et al., 2014). *In situ* baiting is probably the most frequently deployed and involves placing baits directly within the bodies of water being monitored. Examples include floating bait leaves (Huai et al., 2013; Matsiakh et al., 2016), or fruits (Rodríguez et al., 2021) on pond, stream, or river surfaces for 3-10 days before collection and isolating from lesions. *Ex situ* baiting, on the other hand, involves the placement of baits in collected and contained samples away from

the sampling location, ideally under standardized incubation conditions to encourage zoospore release/re-release and infectivity (Sarker et al., 2021). Redekar et al. (2020) used *ex situ* baiting as a method for viable propagule capture as part of their sampling routine for metabarcoding analysis of irrigation water at a horticultural nursery in southern California, USA. They placed fresh leaves of *Rhododendron catawbiense* 'Grandiflorum' in 1L water samples and extracted DNA for analysis from lesions formed following incubation for 3 days at 18-22°C. Looking for lesions on harvested baits moves from using baits for capture to the first stages of detection especially of potentially phytopathogenic oomycete species. Only selecting bait lesions runs the risk of bias in favor of detecting only species capable of rapid infection and symptom induction in the bait species used, and when assessed, asymptomatic baits are often found to have captured a wider range of oomycete species (Sarker et al., 2023b). *In situ* and *ex situ* baiting of bodies of water differ both temporally and volumetrically. They are temporally different in that *ex situ* baits test samples taken from locations at specific times that consequentially represent very short time periods ('freeze frames'). On the other hand, *in situ* baits might be considered to be sampling their locations for as long as they remain *in situ*, although their attractiveness and capacity for inoculum capture will likely vary over time depending on their physical condition. Being limited to restricted to pre-set sample volumes, *ex situ* baiting differs volumetrically to *in situ* where baits are likely to be capturing inoculum from unconfined and, depending on the movement of the sampled water body, more variable and potentially of much larger volume. In soils, recent work has shown that *ex situ* baiting of many small samples as opposed to the generally-preferred large pooled samples can increase the diversity of oomycete species captured (Sarker et al., 2023a; Sarker et al., 2023b). This result is thought to be due to the dilution effect of smaller samples reducing the probability of slower-growing or less aggressive species being out competed in bait infection. A similar effect might be observed in *ex situ* baiting of water samples where it is often assumed that the bulk of inoculum present is motile and infective already and not subject to the limitations of sporulation and release associated with soil. Nevertheless, variation in the aggressiveness of this water-borne inoculum is likely. Additionally, possible bait-induced release of zoospores from caducous sporangia and viable cysts and even the activation of mycelium or oospores in debris, make similar studies with *ex situ* water baiting worthy of investigation.

In soils, the rates of sporangium formation and zoospore release of different species of *Phytophthora* have been demonstrated to affect the efficacy of their detection by baits (Sarker et al., 2021). This process might be reflected in water, where inoculum release from infected plants is often in short-term surges (Hallett and Dick, 1981; Pettitt et al., 2015). These inoculum surges are often initiated by changes in the environment such as increased irrigation frequency or by rainfall (Ristaino, 1991; Café-Filho et al., 1995), but can also result from subtle changes in cultural practice, for example sudden reductions in the root zone temperature in hydroponics crops (Kennedy and Pegg, 1990). There are large seasonal variations in the numbers of oomycete propagules seen in irrigation water in the UK (Pettitt et al., 2015), some likely driven by seasonal changes in water temperature (Pettitt and Skjøth, 2016). A fairly consistent double peak in general oomycete CFU counts is seen in clinic samples from UK nurseries, with larger counts in late Spring and in late Summer-Early Autumn and a decline in mid-Summer (Pettitt et al., 2015). This result is in contrast to total filamentous fungal counts and the numbers of *Fusarium* CFU, which generally reach a single peak in August/September. A similar distribution with distinct peaks of detected CFU in Spring and Autumn was observed in citrus orchard soils (Cacciola and Magnano di San Lio, 2008), and interestingly this represents the annual progress curves of two different species of *Phytophthora* (*P. citrophthora* and *P. nicotianae*).

2.1.2 Filtration

The main alternative to using baits for capture, is to extract oomycete propagules from water by filtration. This approach was initially developed as a precursor to plating viable propagules onto semi-selective agar plates for colony-forming unit (CFU) counts (Ali-Shtayeh et al., 1991; Hong et al., 2002; Pettitt et al., 2002). Filtration to capture dispersed inoculum in water is now also used, often in parallel with baiting, to capture oomycete biomass ready for detection and identification by DNA extraction and nucleotide assays, especially with the increasing use of metabarcoding techniques (Scibetta et al., 2012; Català et al., 2015; Green et al., 2021). To capture viable propagules for colony-plating, membrane filters are used, most frequently of pore size 3 or 5 μm and 47 mm diameter. Once filtration has been completed, membranes can be plated directly onto selective agar media (Davidson et al., 2005; Calvo-Bado et al., 2006; Davidson et al., 2008). Alternatively, the inoculum caught on the filter membrane surface can be released into a small volume of a resuspension medium consisting of 0.09% agar solution (Ali-Shtayeh et al., 1991), ideally containing selective antibiotics at the same concentrations as the plating medium (Pettitt et al., 2002; Büttner et al., 2014). A membrane fabric that readily and reliably releases caught viable propagules is desirable for colony-plating procedures. Hong et al. (2002) investigated a range of membranes and found the most efficient recoveries in terms of filtration speed and colony recoveries were on 5 μm polyvinylidene fluoride ('Durapore 5') membranes, although the only cellulose nitrate filters they investigated were of 0.45 μm pore size, which was the size and type originally used by Ali-Shtayeh et al. (1991). This pore size was originally used at Horticulture Research International Efford (UK) but was found to be too fine and too readily blocked for efficient extraction of oomycetes from irrigation water samples. An increase in pore size to 5 μm cellulose nitrate (Whatman International Ltd, Maidstone, UK) gave faster filtration and consistently high colony recoveries of *Phytophthora* and especially *Pythium* zoospores (Pettitt et al., 2002). The pore size was later reduced to 3 μm following the findings of Hwang et al. (2008), although later comparisons in 2012 between 3 and 5 μm cellulose nitrate membranes and 3 μm polycarbonate (Cyclopore[TM] track-etched, Whatman International Ltd, Maidstone, UK) membranes showed no significant differences in colony recoveries from irrigation reservoir samples (Pettitt et al., 2015).

When filtration has been deployed for the capture stage for genomic studies of oomycetes in water, effective use has been made of mixed cellulose ester filters of pore size 1.2 µm to capture target biomass (Scibetta et al., 2012), although again this size has proved too fine for efficient filtration of field samples, and in more recent studies an increased pore size of 5 µm has been used (Mora-Sala et al., 2022). Interestingly, these workers refined their oomycete extractions by using a baiting technique, placing filter membrane halves into longitudinal cuts in apple fruits, a widely used bait, especially for baiting *Pythium* and *Phytophthora* species from soil samples and decaying, diseased plant tissues (Campbell, 1949). Cuts containing membrane halves were then sealed with parafilm, and molecular identifications of cultures isolated from the resulting lesions were carried out.

Membrane filtration of water samples taken to the laboratory is normally carried out using bottle-top filter funnels such as the reusable, readily-cleaned, and autoclavable Nalgene™ Polysulfone units (Pettitt, 2016), and passing water through the filter using suction from a vacuum pump. In the field, in the absence of an available electricity supply, this method can still be applied using a hand-held suction pump (Matsiakh et al., 2016; Pettitt et al., 2018). Hand-operated suction pumps are compact and work well, but are tiring to the forearms of the operator after a short time. An alternative approach developed by Scibetta et al. (2012) is to push the water through the filter mounted in an in-line polypropylene filter cartridge using an adapted knapsack sprayer (Cooper Pegler CP3). This method is effective, especially for filtering larger volumes of water (2-5 L), although cleaning the apparatus between samples, particularly when collecting samples for eDNA analysis, is time-consuming and requires a comparatively large amount of oomycete-free water. A refinement of this basic concept was developed during the 'Phytothreats' project (Green et al., 2021), using a cyclist's track pump to apply air pressure to a simple pressure vessel that then drives one or more water sample(s) from their connected collection bottles out through up to 3 parallel filter cartridges. This process greatly reduces potential cross-contamination and the of cleaning needed between samples is reduced to just the filter cartridges and their supply manifold, which can be flushed through with cleaning agent and then rinsed with small volumes of sample water before new filter membranes are mounted.

There can be discrepancies in the numbers and abundance of oomycete species captured by filtration and baiting methods (Sarker et al., 2023a), especially with *ex situ* baiting which in comparisons, tends to capture fewer species and less biomass than filtration (Pettitt et al., 2002; Redekar et al., 2020). Recent studies looking at assay conditions and sample sizes for *in situ* baiting of soils are closing this gap (Sarker et al., 2023a; Sarker et al., 2023b), but further studies are required to fully understand the differences seen in water tests.

2.2 Detection and identification

Sensitive techniques for the detection of oomycete propagules have been available for some time (Pettitt et al., 2002; Cooke et al., 2007; O'Brien et al., 2009). However, with the development of new techniques the capacity for rapid, accurate, and precise identifications has increased greatly in the last ten years (Figure 1), especially with the increasing availability of metabarcoding technologies (Burgess et al., 2022). Techniques that can be used for detection and identification fall into three main groups: cultural, immunodiagnostic and nucleotide-based assays.

2.2.1 Culture-based detection and identification

Culture-based detection and identification techniques either consist of plating of captured propagules from baits or directly from membrane filters onto semi-selective antibiotic-amended agar (Tsao, 1970), or of direct observation of signature symptoms and/or sporulation on baits. Depending on the numbers of propagules present in a sample and the condition of the water, culture-based techniques can be highly sensitive with membrane filtration-colony plating potentially resulting in one colony per viable spore (Pettitt et al., 2002). Rapid identifications to genus, either directly from observations of colony morphology on agar plates (O'Brien et al., 2009) or from sporulation on infected baits, are possible (Werres et al., 2014). Culture- plating methods tend to lack specificity and this can be increased by altering the components of the of the mix of fungicides and antibiotics used in the semi-selective medium. Additions of hymexazole to media improves selectivity for many *Phytophthora* spp. (Tsao, 1983; Erwin and Ribeiro, 1996), but this selectivity often comes at the price of reduced sensitivity as a consequence of inhibitory effects of the antimicrobial mix (Tsao, 1970; Sarker et al., 2020). Nevertheless, isolations and/or further culturing to encourage sporulation and properly observe colony morphology are, more often than not, required to achieve a diagnosis using identification keys, a process requiring patience, practice and skill. Nevertheless, extractions of detected oomycetes can be made from colony picks or from recovered baits for examination and further identification by either immunodiagnostic or nucleotide-based assays.

2.2.2 Immunodiagnostic assays

Baiting techniques can be used as a capture and initial detection step prior to testing by enzyme-linked immunosorbent assays (ELISA) or lateral flow devices (LFDs) (Wedgwood, 2014), whilst immunodiagnostic dipstick assays can carry out the baiting capture step without the need for live plant tissues and on collection can progress straight to a diagnostic staining process (Cahill and Hardham, 1994b; Wilson et al., 2000). Immunodiagnostic assay, both ELISAs and LFDs, have also been used directly on extracts of inoculum captured on membrane filters with some success (Wedgwood, 2014). Trapped propagules can also be subjected to direct immunostaining procedures *in situ* on a membrane filter and observed by stereo microscope or a hand lens using the zoospore trapping immunoassay (ZTI), a procedure whereby captured, viable zoospores and cysts are encouraged to germinate *in situ* prior to immuno-staining (Wakeham et al., 1997; Pettitt et al., 2002). The range of immunodiagnostic procedures of potential use in the testing and monitoring of

irrigation water for oomycetes has been more fully reviewed by O'Brien et al. (2009) and more recently by Wakeham and Pettitt (2017).

2.2.3 Nucleotide assays

In recent years, molecular nucleotide assays essentially based around polymerase chain reaction (PCR) have become the preferred method for laboratory-based detection and identification of oomycetes (Schena et al., 2008; Wakeham and Pettitt, 2017: La Spada et al., 2022). For detection of oomycetes in water, PCR tests are used in conjunction with either a filtration or baiting capture technique, and baits have been widely use as a 'biological amplification' stage (e.g., Shrestha et al., 2013; Sena et al., 2018: Kunadiya et al., 2019). Over the last 20 years, basic PCR, quantitative PCR and isothermal amplification techniques have been improved, tested, and refined for oomycete detection and identification. Much of this work has focused on *Phytophthora* because of both the importance of this genus commercially, and the devastating impact of species like *P. cinnamomi* (Carter, 2004; Shearer et al., 2004; Scott et al., 2013; Hardham and Blackman, 2017) and *P. ramorum* (Rizzo et al., 2005; Grünwald et al., 2008) on the environment. There are some excellent reviews of these successful, still developing methodologies (Lévesque and DeCock, 2004; Cooke et al., 2007; O'Brien et al., 2009; Robideau et al., 2011; Martin et al., 2012; Schroeder et al., 2013; Magray et al., 2019).

Using DNA sequence data is a precise method of achieving identifications, but there is sometimes a concern about accuracy in that there are many misidentified sequences in public sequence databases, for example for certain species of *Phytophthora* (Abad et al., 2023). New identifications/diagnoses should be carried out with caution and, where possible, be based on both molecular and morphological characters with close alignment with ex-type sequence data before assigning to species. A good example of this practice is illustrated in the protocol described by Mora-Sala et al. (2022), where new isolates were assigned to a species when the identity was above the 99% cut-off in respect to the ex-type isolates recommended by the *IDphy* online resource (see Abad et al., 2023).

The large amounts of oomycete genomic data that have been generated, the protocols and PCR primers used to access and collate it, and the identification of so many new taxa (e.g. over 100 new *Phytophthora* species alone in the last twenty or so years) can be bewildering to the non-specialist. Nevertheless, robust oomycete phylogenies are now building on the early molecular studies of Cooke et al. (2000) and Lévesque and DeCock (2004). These phylogenies consider both morphological and physiological traits as well as nucleotide sequence data. In addition to the originally-used Internal Transcribed Spacer (ITS) region sequence data from other DNA regions including mitochondrially encoded cytochrome oxidase, cox I and II (Martin and Tooley, 2003; Robideau et al., 2011) and β-tubulin (Villa et al., 2006) are now used. Rigorous databases for this information have also been established to guide accurate species identifications for *Phytophthora* at least (e.g. The *Phytophthora* Database: http://www.phytophthoradb.org, *IDphy*: https://idtools.org/phytophthora/).

More recently, increasingly widespread use of metabarcoding technologies has greatly broadened the scope of oomycete community studies and has been successful in a number of studies looking at either oomycete (Redekar et al., 2019; Redekar et al., 2020; Foster et al., 2022) or primarily *Phytophthora* species (Català et al., 2015; Redondo et al., 2018; Riddell et al., 2020; Green et al., 2021) in water. The wider metagenomic approach also allows broader communities to be considered, meaning that oomycetes in water can be assessed within a broader context (Marčiulynas et al., 2020), an area that needs further exploration. The different 'next generation' sequencing (NGS) technologies and the interpretation software used in metabarcoding techniques are beyond the scope of this review. Each method has its own rates of error, and balancing the use of these together with determining the most effective sequence information to read for accurate species identifications is an ongoing, rigorous process of analysis and critique (Riddell et al., 2019), and as more studies are reported, comparisons and improvements can be shared (Burgess et al., 2022). The NGS metabarcoding approach allows the spectrum of species present in samples to be determined and therefore the possible interrelationships to be explored in a previously-unfeasible context. As broad sweeps are possible, samples can be screened for both familiar endemic and novel species all at once. This capacity makes these methods powerful tools for identifying, monitoring, and tracing new and potentially invasive disease threats. Indeed, a large proportion of oomycete studies testing water samples reported so far have, rightly, been primarily focused on biosecurity.

2.3 Viability

Once oomycete propagules have been captured from a water sample, detected and identified, their viability generally needs to be established. The question of propagule viability is of crucial importance in epidemiological studies, and for determinations of immediate disease risks, as well as for assessments of potential management and control measures. Culture plating, including CFU counts and baiting techniques, detect only viable propagules. With appropriate bait selection, an indication of infectivity is also possible (Werres et al., 2014; Garbelotto et al., 2021) and in some cases this can be confirmed by rapid sporulation for example on excised lupine radicals (Pettitt et al., 1998). Dipstick assays also attract viable spores by chemoattraction (Cahill and Hardham, 1994a). The resulting zoospore cysts that form on the dipstick membrane surface can be immediately subjected to a specific immunostaining procedure, or they can be encouraged to germinate on the membrane first in the same way as in zoospore trapping immunoassay (ZTI), thereby reaffirming their viability (Pettitt et al., 2002; Bandte and Pettitt, 2014). ZTI can measure spore viability and give an indication of the predominant inoculum type in water samples by microscopy within 5 hours of sampling (Wakeham et al., 1997). ZTI assays often require pre-filtration to remove debris, and the immunostaining procedure is more prone to

interference than on dipsticks, especially when turbid samples are being assessed. ELISA and LFD test strips are not able to discriminate between viable and non-viable material (Wakeham and Pettitt, 2017). Whilst limited success has been achieved by coupling these procedures with a baiting step, there is a danger of false-positive tests as antigenic material has been found to attach to bait tissues in inoculated irrigation water previously subjected to heat, chlorination, or UV sterilization treatments (Wedgwood, 2014). Baits have been used as a biological amplification step in ELISA tests of soils (Yuen et al., 1993), providing both a boost in detectable analytes and an indication of inoculum viability. Unfortunately, these benefits come at the cost of reducing the assay to providing qualitative to semi-quantitative estimates of inoculum concentrations.

CFU counts can be highly sensitive measures of viability (Pettitt et al., 2002) and are especially useful in determining the efficacy of water treatments to eliminate oomycete pathogens. Cayanan et al. (2009) used CFU counts to determine the impact of chlorination (Sodium hypochlorite) treatments on the viability of *Phytophthora infestans*, *P. cactorum* and *Pythium aphanidermatum* zoospores in irrigation water. Despite the sensitivity of CFU plates, they lack specificity as indicated above, sometimes making determinations of target viability difficult. Nevertheless, this lack of specificity can also be useful, for example in monitoring the efficacy of water treatment systems such as chlorination or slow sand filtration when installed on nurseries. In these situations, the populations of target pathogen propagules are likely to be very low or hopefully non-existent, and the spiking of the irrigation system with pathogen inoculum is not an option. Using total oomycete CFU counts on a semi-selective agar can therefore exploit the background populations of saprotrophic zoosporic oomycete species generally common in even the 'cleanest' irrigation systems as indicators of water-treatment efficacy (Büttner et al., 2014).

Despite the considerable power of PCR methods for detection and identification, they only detect and amplify pathogen DNA for sequencing and identification and do not differentiate the origin of DNA, or between viable and non-viable tissues (Nielsen et al., 2007; O'Brien et al., 2009; Lau and Botella, 2017). For many applications, this situation is probably of little consequence, as presence of detectable and identifiable DNA indicates presence of the identified species and this information is sufficient. However, in recirculating irrigation water that has been treated to control oomycete propagules, there will likely be many dead pathogen cells and particles of debris present that would not be discerned from viable cells by PCR (Stewart-Wade, 2011), leading to misleading false positive tests. The question as to whether detected DNA represents viable propagules has been directly addressed by two methods: a) the use of the DNA-intercalating dye propidium monoazide (PMA) and b) by extracting RNA and converting it to PCR-measurable cDNA using reverse transcriptase (RT-PCR). PMA cannot enter viable cells and binds with exposed DNA in dead cells by a photo-induced reaction that renders it insoluble and un-extractable (Nocker et al., 2006), while RNA is considerably more labile than DNA and unlikely to remain long in dead tissues (Vettraino et al., 2010; Chimento et al., 2012). Whilst very promising, both methods still present considerable challenges,

sometimes producing seemingly contradictory findings. For example, *P. ramorum* RT-PCR sometimes gave positive tests when culture methods were negative (Chimento et al., 2012), apparently detecting dormant inoculum. However, in *P. cinnamomi* (Kunadiya et al., 2019), the opposite appeared to be the case, possibly as a result of minimal RNA expression (Rittershaus et al., 2013) in truly dormant tissues. Such differences may always pose problems, and while they are problematic for development of reliable and economic screening procedures, they might possibly be examined together with changes in infectivity of baits and the appearance of CFUs to further our understanding of the cycles of pathogen dormancy and activity.

2.4 Quantification

Once detection, identification, and viability have been established, it is important to establish the sizes of the populations present in the water being monitored to determine species prevalence (Zappia et al., 2014). While there are plenty of data reporting presence/absence of species, there is very little reliable data published on infective propagule numbers and how these relate to potential disease outcomes (Hong, 2014). Of the detection procedures outlined so far, baiting and basic PCR provide only qualitative data on presence/absence of species. Dipstick tests readily give propagule counts and can be calibrated to give quantitative estimations of the numbers of spores in water volumes being tested (Bandte and Pettitt, 2014). The specificity of these tests and of ELISA assays, which can also give good quantitative estimates of target organism biomass (although not of viability), is reliant on the specificity of the antibodies used, most of which are likely to cross-react with some non-target species (O'Brien et al., 2009). The specificity of ELISA can be improved by deploying more than one antibody with different cross-reactivities in a double antibody sandwich (DAS-ELISA, Fang and Ramasamy, 2015), although this is still unlikely to achieve complete species specificity (Pettitt et al., 2018). CFU counts can be very precise but, when used alone, lack specificity and require experience and skill in identifying colony morphology for their interpretation (Hong and Moorman, 2005). Nevertheless, CFU counts have been effectively used in combination with PCR to generate valuable epidemiological data on viable spore numbers (Davidson et al., 2008), and if examined early enough, identification of colony origins can be attempted (Pettitt and Pegg, 1991).

Pathogen biomass can be estimated by measuring the concentration of DNA in a sample using 'real-time' or 'quantitative' qPCR (Schaad and Frederick, 2002). qPCR assays have been developed for diagnosis and estimating biomass of a wide range of oomycete species (Cooke et al., 2007; Huang et al., 2010; Tuffs and Oidtmann, 2011; Lees et al., 2012; Strand et al., 2012; Mulholland et al., 2013; Li et al., 2014). Once optimized, qPCR assays are fast, sensitive, and have good specificity, although like conventional PCR they cannot discern viable from non-viable DNA. Nonetheless, the technique can be successfully coupled with classical cultural techniques to provide measurement of biomass combined with identifications of the viable species

present for example of oomycetes in ornamental nursery-stock (Puertolas et al., 2021). Both PMA and RT treatments mentioned in the viability section above can be used for PMA qPCR or RT qPCR, both of which are promising techniques but still not robust enough to be deployed for the large field sample runs needed for in-depth population monitoring, for example requiring species specific internal assays to calibrate each run (Pettitt et al., 2023).

Metabarcoding techniques have so far been largely used as taxonomic screening tools for oomycetes in water, detecting and identifying species present in samples to determine the presence and distributions of pathogens and potentially invasive species to assess risks to the environment and crops (e.g. Green et al., 2021; Burgess et al., 2022; La Spada et al., 2022; Mora-Sala et al., 2022),. The species abundance data generated by this process can give an indication of relative biomass (Di Muri et al., 2020), but not of propagules' viability. Also, PCR-based metabarcoding methods can show primer bias depending on the primer sets used (Elbrecht and Leese, 2015; Mendoza et al., 2015; Burgess et al., 2021), possibly affecting the precision of relative abundancy estimates. Baiting has been used alongside metabarcoding methods but more as a complementary sampling strategy and to isolate cultures than to verify viability (La Spada et al., 2022; Mora-Sala et al., 2022). Nevertheless, metabarcoding and other techniques using high throughput sequencing methods 'have emerged as new paradigms' (La Spada et al., 2022). In just a few years, these have greatly increased the depth and richness of our understanding of oomycete communities, and are also potentially providing the basis for new phytosanitary testing repertoires (Rossmann et al., 2021).

3 Applicability of techniques to different categories of water monitoring
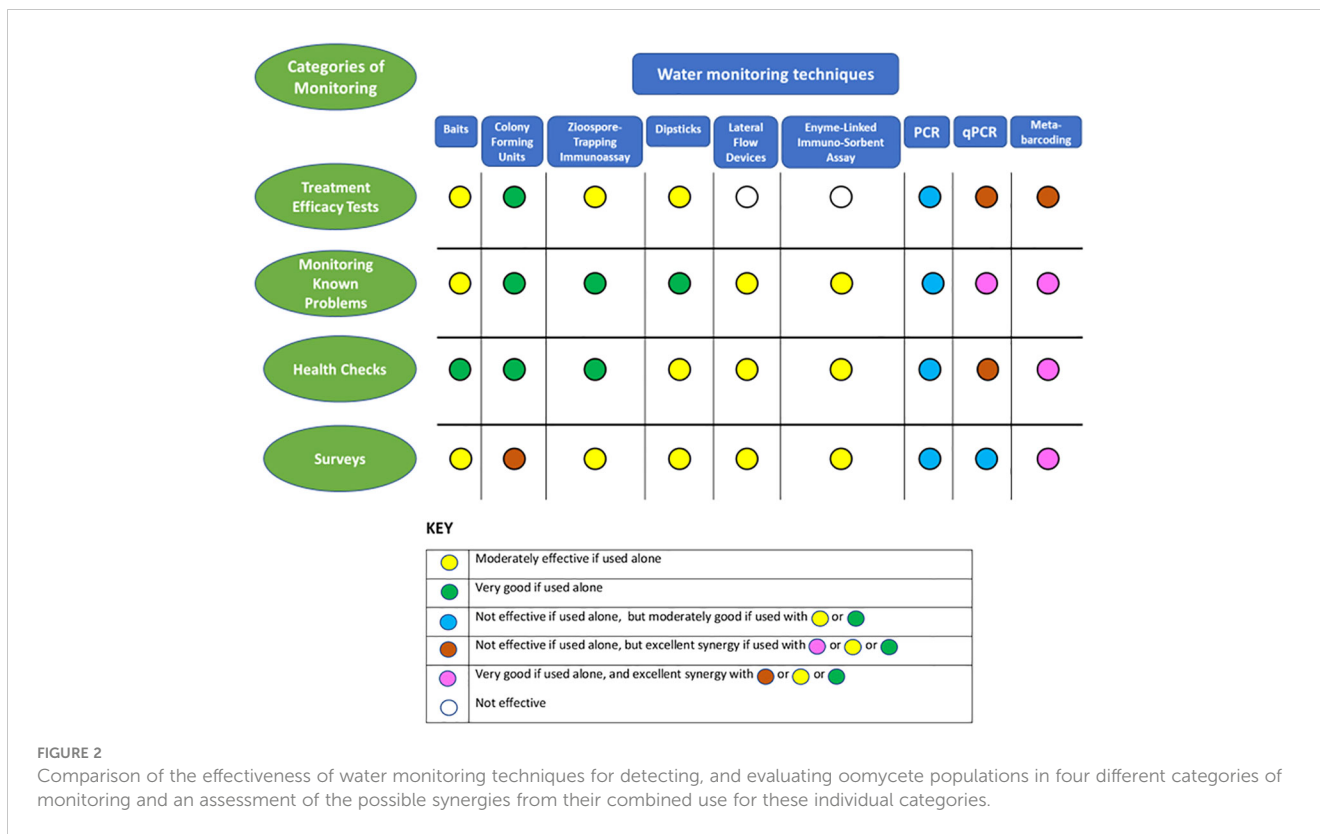
3.1 In general

With the expanded movements of whole plants and plant parts in world trade since the 1990s, there have been rising concerns about invasive pathogens and an increasing awareness of disease risks to both crops and treasured native plants (Brasier, 2008; Fisher et al., 2012). As mentioned above, the oomycetes already have notoriety as a consequence of several plant disease pandemics as well as many invasive disease problems at more localized scales (Brasier et al., 2022). Water has a key role in infection and spread in many oomycete species, and monitoring oomycetes in water is important at different levels of scale; firstly, at a national or international scale to identify sources and pathways of potential pathogen movement and introduction, and secondly, at a local scale at locations such as horticultural nurseries, gardens, parks, forests, and reserves to identify, manage and control disease risks and disease spread. Oomycete monitoring strategies in water address questions of disease risk, but also can address questions about the basic biology and life cycles of these organisms, which in turn contribute to improved

precision of risk prediction and mitigation. Many of the procedures that have been successfully used in monitoring water for oomycetes were initially developed for analysis of soil and plant tissues but inoculum in water tends to be more transient and widely dispersed rather more like airborne inoculum and some aspects of oomycete survival and spread such as cyst formation and polyplanetism have received comparatively very little study. In the first part of this review, the techniques currently used for preparing and analyzing water samples have been considered in relation to a basic framework of monitoring stages: 'capture', 'detection and identification', 'viability testing' and 'quantification'. This second part of the review looks at some of the key kinds of questions likely to be asked under different monitoring categories or scenarios and how the framework shifts with different emphasis placed on these stages and therefore the possible strengths of particular techniques under these different circumstances. This subtle shift in emphasis attempts consideration of the most appropriate methodologies from those outlined in section 2 above for various scenarios based on efficacy, economy of effort and levels of accuracy and precision needed for the nature and circumstances of the monitoring questions being asked. For example, the question 'what are the limits of this water treatment's efficacy?' will require a quite different approach to 'does this water source contain potentially infective oomycete species?'. The former question requires high sensitivity of capture, detection and accurate determinations of viability, but not necessarily high species specificity. On the other hand, the latter question requires the ability to detect a wide range of species with high specificity without being immediately concerned with precise estimates of viability. Despite the recent and ongoing exciting developments with metabarcoding and high-throughput sequencing technologies in general, the best results for many applications still come from complementary use of techniques, especially when estimations of viability and the collection of live voucher specimens are needed (Marano et al., 2012; La Spada et al., 2022).

3.2 Water monitoring categories

Four categories of monitoring are considered here, based in each case on what is already known and the specific questions being asked. The first three categories are site-focused (e.g. park or horticultural nursery *etc.*): 1) water treatment efficacy testing; 2) recognized problem-specific testing (e.g. a recognized and diagnosed recurrent disease) – monitoring epidemics in field situations or within controlled, perhaps inoculated experiments and trials; 3) 'health checks' - testing storage tanks, gutters, irrigation lines and similar structures for contamination; 4) surveys – testing water sources from collection ponds, rivers, open reservoirs, and lakes to puddles. This last category of testing could be site-focused, or consist of surveys of many locations, possibly over wide areas. Water testing techniques for oomycetes suit these categories of monitoring to different degrees, and often there are synergies to be gained from combining quite different techniques within a monitoring strategy (Figure 2).

FIGURE 2
Comparison of the effectiveness of water monitoring techniques for detecting, and evaluating oomycete populations in four different categories of monitoring and an assessment of the possible synergies from their combined use for these individual categories.

3.2.1 Water treatment efficacy testing

For the first category of monitoring, probably the best approach to assessing the efficacy of water treatments *in situ* is the use of resuspension-colony plating and counting CFU (Büttner et al., 2014), as there is a need for sensitivity but not for a high degree of specificity. Treated and untreated water samples could also be sensitively monitored using ZTI or immuno-dipstick assays using more generic anti-oomycete antibodies than those used by Cahill and Hardham (1994a) or Gautam et al. (1999), (Pettitt et al., 2002).

3.2.2 Local problem-specific testing

The second category of monitoring presents a more complex situation regarding the need for an initial diagnosis for *in situ* monitoring programs, which would pragmatically still involve a combination of cultural and molecular (metabarcoding) approaches to cast a broad net and minimize presumptive bias (La Spada et al., 2022; Mora-Sala et al., 2022; Sarker et al., 2023a). Depending on the complexity of the diagnosis, it may then be possible to proceed to quantification of inoculum in relation to the specific problem. Again, CFU counts can be used here if colony morphologies are distinctive enough, and ZTI or immuno-dipstick approaches may be workable, depending on the specificity of available antibodies. In many instances in this category of monitoring, it may not be essential to continuously monitor viability. In this case, depending on the availability of suitable primers, qPCR or even multiplex qPCR (Schena et al., 2006) would provide excellent, specific determinations of inoculum concentrations in samples. The assumption here is that this type of monitoring would require large numbers of samples, and currently the costs of using

a metagenomics approach for this may prove cost-prohibitive. However, this situation should change as costs will likely reduce with the pace of developments.

3.2.3 'Health checks'

The third category of monitoring is generally concerned with sites like horticultural nurseries or gardens that can have complex irrigation rigs (or maybe just gutters for collecting rainwater) that need to be routinely examined for oomycete and other potential pathogens. This is a situation where metabarcoding, combined with either baiting or filtration-colony plating, would provide excellent information, but at the moderate to high intensity of sampling needed, again, might not be economic. Currently, this kind of monitoring is carried out using the same kind of culture-based methodologies as the first category of monitoring, often with the inclusion of generic culture media such as basic potato dextrose agar in addition to oomycete-selective media to crudely assess the 'background' microbiota as well as oomycetes (Büttner et al., 2014).

3.2.4 Surveys

The fourth category of monitoring was, for a long time, only feasible using baiting and culturing methods (Erwin and Ribeiro, 1996). These were later supplemented and greatly improved by augmenting morphologically-based identifications with PCR and sequencing (Ghimire et al., 2009; Ghimire et al., 2011; Riolo et al., 2020). This methodology was further adapted for the monitoring of *Phytophthora* species in water by the extraction and nested PCR procedure of Scibetta et al. (2012) and has subsequently been revolutionized by the use metabarcoding and metagenomics

procedures which offer high levels of accuracy, sensitivity and some measure of abundance or relative abundance of detected taxa (Green et al., 2020; Green et al., 2021).

3.3 Future possibilities

There is still much to learn about the ecology and diversity of oomycete species in water and despite continued enormous improvements in monitoring techniques there are still gaps in our knowledge especially relating to prevailing propagule types, their behavior and survival in different types of water environment. Culture-based and immunodiagnostic approaches to monitoring allow some level of propagule assessment and give the most reliable determinations of viability and potential aggressiveness, but lack specificity. DNA-based techniques provide high levels of specificity and with the ongoing improvements in the efficiency and economy of high throughput sequencing technologies, the levels of detection sensitivity and identification specificity and reliability using metagenomic techniques are set to rise. Improvements in analysis of results may also come from software advances, whilst prospecting for addition metagenomic loci to try and improve discrimination of specific taxonomic groups of interest. Also, more basic improvements may well be possible, such as the honing of DNA extraction techniques specifically for oomycetes from water samples or the adaptation of centrifugation techniques used for virus capture (Büttner et al., 2014), possibly to improve effective oomycete capture from more turbid water samples. More conventional methods still also have the potential to be honed for the monitoring of water samples, for example modifications to selective agar media for culture plates are capable of improving selectivity, whilst ongoing studies with soils show possibilities for the improved use of baiting techniques (Sarker et al., 2021; Sarker et al., 2023a; Sarker et al., 2023b).

There are further useful examples for potential future water studies from work in soils, for instance changes in fungal and oomycete community composition have been successfully monitored using a metabarcoding approach (Del Castillo Múnera et al., 2022) which demonstrated significant reductions in the abundance and diversity of oomycete pathogens in the rhizosphere of poinsettia crops as a result of reduced irrigation. Other studies show the power of these techniques: for example, in studies of fungal and oomycete communities in Holm oak soil, Ruiz Gómez et al. (2019) examined some complex interactions and identified a strong correlation between the presence of a taxon of *Trichoderma* and a decline in the abundance of pathogenic *Phytophthora* spp.

This exciting work still needs to be complemented by the use of more established approaches, especially cultural techniques. Techniques such as baiting (e.g. La Spada et al., 2022; Mora-Sala et al., 2022; Sarker et al., 2023a), or possibly CFU plates (Davidson et al., 2008), are still key to establishing viability, testing pathogenicity and infectivity, examining morphological characteristics, and obtaining voucher specimens. Nevertheless, metabarcoding/metagenomic studies have obvious application to the monitoring and understanding of oomycete communities in free water, and possibly with the use of autonomous sampling technologies (Searcy et al., 2022; Truelove et al., 2022), a far greater understanding can be achieved of the spread of these specific inocula in time and space. These possibilities show the way for future work with sampling in depth and over time to evaluate and mitigate the risks of disease, and local and international pathogen spread.

Author contributions

The author confirms being the sole contributor of this work and has approved it for publication.

Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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