Differentiation of environmental aquatic bacterial isolates by MALDI-TOF MS

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Abstract

Identification of bacteria in aquatic and environmental applications, for monitoring purposes and research, for health assessments and therapy considerations of farmed and free-living aquatic organisms, still relies on conventional phenotypic and biochemical protocols. Although molecular techniques based on DNA amplification and sequencing are finding ways into diagnostic laboratories, they are time-consuming, costly and difficult in the case of multiplex assays. Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) is a rapid and accurate proteomic method reliable for identification of unknown bacteria to the genus and species level. Upon extension of databases, it will certainly find its position in environmental sciences. The paper presents an overview of the principle of the method, its effectiveness in comparison with conventional and molecular identification procedures, and applicability on environmental and aquatic isolates, discussing its advantages and shortcomings, as well as possible future implementations.

Keywords:

MALDI-TOF MS, environmental bacteria, fish, crayfish

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1. Introduction

The broad microbial biodiversity in aquatic environments fluctuates in respect to environmental conditions, differing from one aquatic macroenvironment to another, as well as in various sublocations in the same aquatic macroenvironment. Microorganisms are integral components of all aquatic ecosystems, and they are essentially responsible for biogeochemical cycles and key environmental processes (Amalfitano et al., 2014). In association with organisms living in waters, they exist mostly as symbionts and sometimes as pathogens. Therefore, fish, crayfish and other aquatic organisms are susceptible to a variety of bacterial infections, some of them host specific, some general, and some associated with immunosuppressive impact of water pollution (Austin and Austin, 1999). They can be either pathogenic to wild and cultured species or pose a threat for outbreaks of diseases under favorable conditions. Some bacterial fish pathogens may also infect humans and cause zoonotic diseases, mostly in immunocompromised patients. Consequently, it is apparent that the attention has to be devoted to rapid and accurate identification of environmental bacterial isolates.

Most methods for bacterial cultivation and identification are still based on the application of general and/or specific culture media, standard morphological, physiological and biochemical tests and their comparison to standard results. Routine steps for culture and identification of an organism, from sample collection and preparation to the interpretation of biochemical results from appropriate identification tables, usually take up to five days, or 96 hours (Buller, 2004). However, prompt and accurate identification is essential in order to be able to successfully cope with disease-causing environmental bacteria. Along with characterization based on phenotypic properties of bacteria, a number of nucleic acid methods have been developed. They alone do not define the bacterium and must be considered together with the phenotypic ones, but are very helpful in better definition of the bacterium. These methods mainly comprise the G + C ratio, DNA-DNA hybridization, ribotyping and fluorescent *in situ* hybridization (Okafor, 2011). The real-time PCR and 16S ribosomal RNA (16S rRNA) sequencing enabled microbiologists to obtain information on bacterial composition without cultivation. Though time-consuming, costly and difficult in the case of multiplex assays, PCR is now a common technique used in medical and biological research (Campos Braga et al., 2013).

However, there are several challenges in identification of environmental, and particularly aquatic bacteria, such as discrimination between closely related environmental strains, need for rapid identification in some disease outbreaks, identification of rare or less frequent microorganisms which are difficult to discriminate with classical techniques. All of these challenges can be successfully tackled with the application of matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS). The principle and advantages of the method will be discussed in this review, as well as the recent breakthroughs, with particular emphasis on its ability to differentiate environmental bacterial isolates.

2. MALDI-TOF MS, the method

The mass spectrometry (MS) for microbial biotyping was initially attempted by analysis of lipid content profiles for some bacterial species (Anhalt and Fenselau, 1975). Other MS methods such as pyrolysis MS (lipid biomarker), fast atom bombardment MS (lipid biomarker), gas-chromatography MS (carbohydrate biomarker) and electrospray ionization MS (protein/peptide

biomarker) were also employed with moderate success (Mazzeo et al., 2006). In the 1980s, the development of soft ionization matrix-assisted laser desorption/ionization mass spectrometry allowed the analysis of relatively larger protein biomarkers (Karas et al., 1985). MALDI-TOF MS for direct recognition of bacterial proteins in whole colonies was introduced in the nineties (Claydon et al., 1996; Holland et al., 1996; Lavigne et al., 2013). Whole or intact bacteria comprise cells suspended in a solution and/or deposited directly on the sample holder, as exposure to solvents, acids or water in the MALDI matrix tends to lyse them (Fenselau and Demirev, 2001). Protein profiles can be obtained from a single bacterial colony directly deposited on the target plate and overlaid with the matrix solution (Seng et al., 2010). According to Emami et al. (2012), the quality of spectra obtained using lysates was higher than those form intact whole cells, with the advantage that the instrument and supplies would not be contaminated if using microbiologically sterile lysates.

The method detects mainly the most abundant and conserved ribosomal protein fractions of bacteria which can be used for classification of the organisms (Lay, 2001). The bacterial identification is based on the detection of mass signals from proteins that are specific at genus, species or sub-group levels (Benagli et al., 2012). Measured mass signals are compared with mass spectra for referent bacterial strain collected in dedicated mass spectra library (library based approach) or with publicly available proteomics/genomics data (bioinformatics enabled approach) (Sandrin et al., 2013). Bioinformatics enabled biotyping usually require accurate peptide mass measurement and mass spectrometers for these experiments are equipped with a mirror which reflects ions using an electric field, thereby doubling the ion flight path and increasing the resolution and mass measurement accuracy. Time of flight of the ions in gas phase is relative to a mass/charge ratio (*m/z*), and ions with a high *m/z* value fly slower then ions with a low *m/z* value. Due to the possibility to ionize and introduce non-volatile molecules into the mass spectrometer, the method enables classification and identification of microorganisms (Risch et al., 2010). The mass spectra are thus considered specific fingerprints or molecular profiles of bacteria analyzed, and are compared with the spectra in databases developed by various bioinformatic tools (Mazzeo et al., 2006). Different search engines were developed in order to build and search databases of bacterial protein spectra (Bright et al., 2002), as well as fingerprint libraries for mass spectral peaks, typically in the range of 4-15 kDa, with a key requirement of spectral reproducibility (Demirev et al., 1999). Protein databases for bioinformatics enabled approach are based on Swiss-Prot/TrEMBL or NCBInr data and have a low number of protein entries for bacteria of partially sequenced genomes, such as various environmental bacteria (Mazzeo et al., 2006). For commercial biotyper platforms mass spectrometers are supplied with database systems for routine identification of bacteria, with spectra collected from reference strains, containing up to 2000 species with over 3000 spectra (Lavigne et al., 2013).

For ionization of protein samples, the matrix solution is mixed with the analyte, enabling formation of protein spectra with specific molecular weight ranges. The matrix consists of crystallized molecules, and the most frequently used are alpha-matrix (alpha-4-cyano-4-hydroxycinnamic acid, CHCA), sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid, SA), and 2,5-dihydroxybenzoic acid (DHB) (Beavis et al., 1989; Beavis et al., 1992; Strupat et al., 1991). Matrices are often specific to Gram staining of bacteria, therefore allowing identification of mixed cultures with different Gram types (Evason et al., 2001). However, as variations in sample preparation can influence mass spectra, standardized protocols need to be applied in regards to incubation time, type of culture media, matrix, extraction solvent, salt content and deposition method (Ruelle et al., 2004; Emami et al., 2012).

3. Comparison with conventional and molecular identification procedures

The prerequisite for any microbial identification is a rapid and reliable test which can be conducted in various types of clinical, environmental, veterinary and other studies. Tedious biochemical and phenotypical procedures, which use both manual and automated platforms, are utilized alone or in addition to molecular methods. Although some of these tests are straightforward, the complete identification usually takes at least one day, and they often do not provide a reliable answer in routine microbiological identification (Table 1). With MALDI-TOF MS, aside from the easy data analysis, time to result is a few minutes after culture, while running costs are low (Lavigne et al., 2013), esp. when compared with some of the conventional identification systems. For example (in euros/minutes), API systems on average take 4.6-6.0/1080-2880, BD Phoenix 12.65/300-1200, Vitek systems 5.9-8.23/300-480, and MALDI-TOF MS 1.43/6-6,5 (Seng et al., 2009). Costs and time-frames for 16S rRNA analyses may be substantial per isolate, depending on sequences and repetitions (Clarridge, 2004; Justesen et al., 2010). There are a number of available conventional diagnostic microbiological tools which were compared for their identification capacity and reliability with MALDI-TOF MS, mostly for clinically relevant bacteria (Risch et al., 2010; van Veen et al., 2010; Saffert et al., 2011; Alvarez-Buylla et al., 2012; El-Bouri et al., 2012; Fang et al., 2012; Meex et al., 2012; Zangenah et al., 2012; Caretto et al., 2013; Jamal et al., 2013; Almuzara et al., 2015, to name a few recent studies).

Some commercially available diagnostic tests form a part of routine laboratory diagnostics, such as API 20E, API ZYM, API 20NE, API 20AN, API 50 CH, API Rapid ID 32 (bioMerieux, Marcy-l'Etoile, France), Biolog MicroPlates GN2, GP2, AN (Biolog, Inc., Hayward, CA, USA), Enterotubes, BBL Crystal E/NF (Becton-Dickinson & Company, Franklin Lakes, NJ, USA), Bionor Aqua (Bionor, Skien, Norway) systems and some others (Topić Popović et al., 2007). Of these, API 20E rapid identification system has been the most widely used in identification of Enterobacteriaceae and other non-fastidious Gram-negative bacilli. Conventional methods result in fewer species identification and more major errors than MALDI-TOF MS in discrimination of both Gram-negative and Gram-positive bacteria (van Veen et al., 2010). For Gram-positive anaerobic cocci only MALDI-TOF MS allowed accurate identification to the species level (Kierzkowska et al., 2013) when compared to other diagnostic panels. The use of MALDI-TOF MS was reliable in identification of close to 300 colonies isolated through different seasons and various points from water sources, when compared to API-based identifications and even to 16S rRNA gene sequencing (Sala-Comorera et al., 2016). One of the reasons that mass spectrometry outperforms API systems is that the current taxonomic database of the MALDI Biotyper system recognizes species of different taxonomic status that have not been updated in the apiweb database (Kierzkowska et al., 2013). Because of the open platform design, additional spectra can be uploaded to the Bruker Biotyper database, achieving identification of species previously possible only with 16S rRNA sequencing (Saffert et al., 2011). Overall, conventional tests are rather time-consuming metabolic reactions, which require experience with morphological and observer-dependent judgments (Risch et al., 2010). Adversely, MALDI-TOF MS can be used as the initial method for identification, before Gram staining and any biochemical profiling, when using a database which includes 10 reference spectra per bacterial species and an identification score ≤ 1.9 (Seng et al., 2009).

PCR is less prone to errors in comparison to conventional methods for identification of bacteria that rely mostly on phenotypic and morphological features of strains (Campos Braga et al., 2013). However, although DNA is a characteristic biomarker for any microorganism, there is just one copy per cell if not amplified. When analyzing an intact bacterium, it has to be extracted, separated and amplified, and that is why molecular methods are generally considerably more expensive and slower to perform, while proteins provide the most characteristic biomarkers accessible for identification and discrimination of bacteria (Fenselau and Demirev, 2001). In comparison with standard 16S rRNA sequencing, MALDI-TOF MS provided similar results, in some cases superior and enabling a taxonomic classification to the subspecies level (Hausdorf et al., 2013). Often, MALDI-TOF MS and 16S rRNA are run to confirm the identity of the species. In freshwater samples, accurate findings of isolated strains were reported with both tests for waterborne pathogens in aquatic environments (*Salmonella, Legionella, Yersinia, Campylobacter*) and fecal indicator bacteria, including *E. coli* and *Enteroccocus* (Thevenon et al., 2012; Lee et al., 2014). However, MALDI-TOF MS surpassed both phenotypic and genotypic approaches for identification of *Pseudomonas* and *Bacillus* genera, where 16S rRNA identified to the species level only 50 % of strains, while MS fingerprinting identified 76 % (Böhme, 2013). Molecular techniques are needed for identification of species within the *Acinetobacter* genus, although MALDI-TOF MS gives a correct identification of *A. baumanii* (Alvarez-Buylla et al., 2012). Recent findings have, however, revealed that MALDI-TOF MS is unsuitable to discriminate *A. baumanii* clones (Sousa et al., 2015). Non-fermenting bacteria in general (taxonomically heterogeneous genera *Pseudomonas, Burkholderia, Stenotrophomonas* etc.) are frequently misidentified by classical methods, and for them 16S rRNA provides less accurate results at the species level. For that reason, MALDI-TOF MS reference databases for non-fermenters are constantly updated (Campos Braga et al., 2013), giving a high reproducibility for identification of these bacteria, with 98.75 % of correct species identification (LiPuma, 2010). Enterobacteriaceae and *Cronobacter* were assigned 100 % to the species level with both 16S rRNA and MALDI-TOF MS, but the MALDI-TOF MS identifications were more discriminating and unequivocal in the work of Zhu et al. (2011). Earlier studies attributed 16S rRNA gene sequencing the title of a gold standard, although it is known that different sequences within a short base pair stretch may question species alignment based on metabolomic identification (Risch et al., 2010). Furthermore, 16S rRNA is not introduced in routine microbiology laboratories, however MALDI-TOF MS, due to its effectiveness and time-saving properties, is making way to routine laboratory diagnostics.

4. Potential aquatic pathogens

Microbial communities in the environment are extremely diverse; however waters and animals that inhabit them are of particular interest. For example, the etiology of bacterial diseases in wild aquatic animals is often improperly understood, as the study of aquatic animals' diseases mainly concentrates on problems relating to fish farms. The ecology of such pathogens is understudied since it is often unclear whether a bacterium is a representative of the natural aquatic microflora or is restricted to organisms, and what the possible role of pollution in the outbreaks of diseases is (Austin and Austin, 1999). Table 2 lists the most important aquatic bacteria that may be either a part of the normal flora of healthy aquatic species, or pathogenic to their hosts. In stressed animals these bacteria may override their defense mechanisms and cause infections. Most of the

bacteria presented in Table 2 can be found worldwide and some of them have also been isolated from humans.

There is a growing awareness that a number of aquatic bacteria (pathogens and/or commensals) may infect humans and cause zoonotic diseases. Although comprehensive statistics still lack, the number of cases of human diseases traceable to aquatic animals is relatively small (Austin, 2010). Transmission to humans occurs mostly from handling, via wounds or is foodborne or waterborne involving a direct entry into the digestive system (Austin, 2010; Haenen et al., 2013). Of bacteria generally presented as fish related zoonoses, only the following are well supported as zoonoses in the strict sense, causing diseases in both aquatic animals and humans: *Mycobacterium* spp., *Streptococcus iniae*, *Clostridium botulinum*, *Vibrio vulnificus* (Gauthier, 2015). Other reported sporadic human infections are associated with *Aeromonas hydrophila*, *Edwardsiella tarda*, *Salmonella paratyphi* B var. Java, *Grimontia hollisae*, *Photobacterium damselae* subsp. *damselae*, *V. alginolyticus*, *V. harveyi*, *V. fluvialis*, and *V. mimicus* (Austin, 2010; Weir et al., 2012; Haenen et al., 2013). These organisms are widespread in freshwater, estuarine and marine environments, on invertebrates, mollusks, cephalopods, fish and other aquatic animals. New pathogenic bacterial species are frequently reported, thus the range of zoonoses may also increase, requiring prompt and accurate identification and diagnosis, particularly in cases of infections which turn into septicemia or life-threatening conditions.

5. MALDI-TOF MS applicability for environmental isolates (water, biota)

Bacteria represent the largest reservoir of biodiversity to be studied, as they are estimated to number over 10^{30} worldwide (Schloss and Handelsman, 2004). However, less than 1 % of the bacteria from natural habitats can be grown in laboratory (Dykhuizen, 1997). The accuracy and speed in obtaining data makes MALDI-TOF MS a powerful tool of significance for environmental microbiology (Donohue et al., 2006). It has been successfully applied to identification of clinical bacterial isolates, but its application in an ecological context is rare (Hausdorf et al., 2013). MALDI-TOF MS was applied in identification of possible contributing sources to fecal contamination of surface waters (Siegrist et al., 2007). Typically, methods for bacterial source tracking use labor-intensive, time-consuming phenotypic traits or genotypic profiles, but MALDI-TOF MS in the work of Siegrist et al. (2007) discriminated closely related environmental strains of *E. coli* and characterized them according to their respective specific sources, which is essential for safeguarding public health and managing water resources.

A few studies clearly demonstrate the usefulness of rapid discrimination of environmental and particularly aquatic bacteria, mostly *Vibrio* and *Aeromonas* strains (Donohue et al., 2007; Benagli et al., 2012; Eddabra et al., 2012, Schirmeister et al., 2014; Topić Popović et al., 2015). Koubek et al. (2012) recommend whole-cell MALDI-TOF MS over 16S rRNA for rapid screening and discrimination of various environmental bacterial isolates. MALDI-TOF MS was also used to aid in discrimination of two closely related *Vibrio* species, *V. furnissii* and *V. fluvialis*, both emerging human pathogens, associated with consumption of seafood or drinking of contaminated water (Schirmeister et al., 2014). It was also applied in rapid discrimination of environmental *Vibrio* species from wastewater samples (Eddabra et al., 2012), where it proved capable of differentiating all 30 closely related *Vibrio* isolates. MALDI-TOF MS was successfully used for interspecific differentiation of *Vibrio* strains extracted from samples of boat

ballast waters, detecting a complete range of pathogenic and non-pathogenic strains (Telesmanitch et al., 2014).

Of particular interest are aeromonads, potential animal and human pathogens occurring in almost every aquatic environment, as it was found that environmental isolates of *Aeromonas* have a wider phenotypic diversity than isolates from clinical samples (Donohue et al., 2007). In a study investigating the potential for using the *m/z* signature of known *Aeromonas* strains to speciate unknown environmental isolates, the aeromonads atypical of the species to which they were assigned biochemically were not concordant with the MALDI-TOF MS assignment. However, it was shown that closely related genera have unique *m/z* signatures not overlapping with *Aeromonas* strains (Donohue et al., 2007). Although it was indicated that MS can be a very useful tool for aeromonad speciation, as also demonstrated in the work of Benagli et al., (2012), who established that the MS distinguished well between genera, species and aeromonad strains, a later study (Vávrová et al., 2015) found that MALDI-TOF MS is not a reliable diagnostic technique for environmental aeromonads. That was concluded on account of high percentage of false-positive, incorrect, and uncertain identification results, both of newly described aeromonads and of pathogenic ones. To the contrary, Lamy et al. (2011) established that genus-level accuracy of clinical and environmental aeromonads identified by mass spectrometry was 100 %, while species-level accuracy reached 90.6 %. Some *Aeromonas* species are known for their complex taxonomy and limited possibilities of identification to the species level. One of such examples is *A. media*, having a wider *m/z* signature range than other species, thus more strains should be added to the databases to best represent this bacterium (Donohue et al., 2007). In a recent study on aeromonads from treated wastewater and fish, we observed that among 24 genera isolated from water, sludge and fish samples, aeromonads were the most represented group (Topić Popović et al., 2015). Also, the identification match between conventional identification systems and MALDI-TOF MS was also the highest for aeromonads to the genus level, with *A. veronii* as the most frequent aeromonad identified. Over 6 % of *Aeromonas* in our study were identified as *A. bestiarum*, but were assigned as *A. hydrophila* by conventional methods. Excluding the database deficiency, it is possible that this identification disparity can be attributed to close relatedness of the two species, belonging to the same phenogroup, described as the *A. hydrophila* complex (Martino et al., 2011). MALDI-TOF MS can however assure their good discrimination at the genospecies level comparable with the *gyrB* gene sequencing as it clusters well differentiated *A. bestiarum* and *A. hydrophila* (Benagli et al., 2012).

The need for comparison of MALDI-TOF MS data with results obtained from phenotypic and genotypic identification methods is still important for identification of environmental isolates. We confirmed that in our study where we isolated bacteria from tissues of apparently healthy freshwater crayfish (Topić Popović et al., 2014). From 25 relevant isolates, only one matched to the species when compared with conventional methods (*Hafnia alvei*), while API 20E and MALDI-TOF MS identified 60.86 % identically to the genus. The highly probable species identification with MALDI-TOF MS was mostly related to *Pseudomonas*, *Bacillus* and *Hafnia* (Figure 1). The most prevalent genus identified was *Pseudomonas*, one of the most frequently isolated non-fermenters from crayfish, and a potential pathogen. Although half of the isolates were pseudomonads, only 39.13 % were assigned to the species level with both systems. Commercial biochemical test (Rapid 32 ID Strep) was also used in comparison with MALDI-TOF MS in order to identify *Enterococcus* spp. isolated from various water environments, and in 85 % they both gave concordant identification results, while the rest were not detectable by the biochemical system (Taučer-Kapteijn et al., 2013). In discrepant identification outputs, the 16S

rRNA gene sequencing confirmed the MALDI-TOF MS identification. Interestingly, identification of *V. parahaemolyticus* isolated from sea water, shellfish and sediments was consistent through Vitek-2 system and MALDI-TOF MS in the work of Malainine et al. (2013). MALDI-TOF MS was used to further discriminate between closely related environmental *V. parahaemolyticus* strains, and when placed at a 10 % threshold of the whole diversity, isolates differed by at least three mass peaks, revealing a larger biodiversity when analyzed through mass spectra of abundant proteins (Malainine et al., 2013).

Identification of waterborne bacteria by MALDI-TOF MS is more straightforward when conducting identification of microbial pathogens that occur in water, such as *Helicobacter pylori, Salmonella typhimurium, Yersinia enterolitica, Legionella pneumophila,* and *Campylobacter jejuni,* since MALDI-TOF MS identification results were well correlated with 16S rRNA gene sequencing identification in the work of Lee et al. (2014). When differentiating the unknown flavobacteria from freshwater and biofilm samples in order to rapidly group them according to overall phenotypic resemblance, the peak profile of only one *Flavobacterium* species was available for comparison, due to the lack of reference organisms (Brambilla et al., 2007). However, more than 30 strains clustered around this strain at similarity levels up to 50 %, showing rich diversity with peak similarities up to 80 %, and were confirmed with 16S rRNA gene sequencing as genus *Flavobacterium.* Affiliation with that genus on the basis of MALDI-TOF MS was further confirmed with flexirubin and fatty acid analysis (Brambilla et al., 2007). The challenge is also to differentiate between environmental and clinical isolates of the same bacterial species since they, although of the same molecular type, might be markedly diverse in enzyme activities and cytolethality. In order to determine whether MALDI-TOF MS can group *Burkholderia pseudomallei* isolates according to their respective sources (environmental *vs.* clinical), Niyompanich and coworkers (2014), generated a principal component analysis of the raw *B. pseudomallei* mass spectra and a dendrogram, demonstrating that the isolates are intermixed and not forming distinct groups. However, although not clustered according to their sources, most of the isolates were differentiated according to their respective source groups using the whole-cell MALDI-TOF MS by constructing an average mass spectrum of the environmental bacterial set and of the clinical set. The mass spectra of the two groups demonstrated high similarity in peak patterns, but differed in peak intensities (Niyompanich et al., 2014), therefore allowing source differentiation. Thus, examination of isolates in terms of source categorization by MALDI-TOF MS could be altered relative to a variety of tested bacteria.

Grouping of environmental isolates from unknown environments is essential in biodiversity studies where large numbers of strains need to be isolated on different growth media, particularly since easily isolated organisms constitute a minor fraction of total bacterial community in environmental samples (Dieckmann et al., 2005). MALDI-TOF MS was found a powerful tool for a high-throughput dereplication of environmental samples, a process of recognizing identical isolates at a specific taxonomic level and grouping them accordingly (Ichiki et al., 2008; Ghyselinck et al., 2011), Dereplication involves rapid screening of all isolates in order to recongnize and eliminate those that represent the same taxon (Spitaels et al., 2016). Today, MALDI-TOF MS is used as a dereplication tool for grouping isolates at the species level, while groups of spectra are generated mostly using algorithms based on the presence or absence of peaks, or on peak intensities (Spitaels et al., 2016). For dereplication purposes it was first used on bacteria isolated from marine sponges (Dieckmann et al., 2005), and was also successfully applied in extreme surroundings, such as hypersaline environments, where it was used for dereplication of halophilic prokaryotes (Munoz et al., 2011; Viver et al., 2015).

MALDI-TOF MS has the capacity to simplify both identification and dereplication, as mass spectra of isolates that cannot be identified directly can be clustered, and representative bacteria can be subjected to further polyphasic identification (Spitaels et al., 2016; Kopcakova et al., 2014).

At present, MALDI-TOF MS methodology for characterization of bacteria greatly varies through differences in sample preparation methods, matrix solutions, organic solvents, acquisition methods and data analysis methods (De Bruyne et al., 2011). The standard protocols for MALDI-TOF MS must be established in order to reduce data variation between laboratories and assure reproducibility (Niyompanich et al., 2014). Generally, it can be concluded that for aquatic/environmental isolates, MALDI-TOF MS still yields lower identification precision than in routine, expected, clinical samples with defined databases. Although most MS databases relate to bacteria with relevance mostly to the medical field, the number and diversity of reference strains cataloged in the mass spectral libraries are being expanded, particularly in order to precisely identify unknown environmental isolates, and also to minimize the possible overlapping of mass ions from other genera (Donohue et al., 2006; Schumann and Maier, 2014; Zhang et al., 2015), therefore it is expected that applicability of MALDI-TOF MS for environmental isolates will soon reach its full potential.

6. Overview and future prospects

The advantages of the MALDI-TOF MS method for identification of environmental aquatic bacteria are numerous. It is the most promising technology for microbiological analyses. Sample preparation is minimal as it analyzes whole bacterial cells. It provides excellent accuracy and specificity with high throughput, can easily be adapted to bacteriology laboratory workflow, data analysis is easy, time to result is just a few minutes after cultivation of the organism, which reduces the possibility of contamination, while running costs are low (Donohue et al., 2006; Seng et al., 2009; Sauer and Kliem, 2010; Böhme et al., 2013; Lavigne et al., 2013). Databases are constantly updated and the possibilities to identify unknown, rare and fastidious bacteria are increasing. Some limitations of the method also have to be addressed, the most important one still being the incompleteness of databases, which need expansion, especially regarding the environmental isolates (van Veen et al., 2010; Lamy et al., 2011; Kopcakova et al., 2014). There is a need for tested protocols on preparation of fastidious bacteria and optimization of measurement conditions (Schumann and Maier, 2014). Also, identification requires relatively large number of cultivated bacteria (an intact colony), while the methods offers a limited possibility to identify bacteria directly from tissues. The technique is confident with pure colonies, while it is not reliable with polymicrobial material (Lavigne et al., 2013). The reproducibility and accuracy of MS can be influenced by culture media, growth time, bacterial concentration, sample preparation, calibration and matrices (Demirev et al., 1999; Mazzeo et al., 2006; Saffert et al., 2011).

Novel applications of the MS will certainly be a step further in microbiology. Although MALDI-TOF MS is still limited in the number of detectable targets for strain characterization (Karlsson et al., 2015), efforts have been made to increase the taxonomic resolution of the MALDI-TOF MS, using library-based and bioinformatics-enabled approaches to enhance strain categorization, differentiation and identification of bacteria at the strain level. Bioinformatics-

enabled approaches facilitate strain profiling through intact biomarker identification, bottom-up, and top-down procedures, not suffering limitations of culture conditions and sample preparation, thus enhancing the strain resolution possibilities of the method (Fenselau and Demirev, 2001; Kok et al., 2013; Sandrin et al., 2013). Availability of bioinformatics tools for database searches allows not only secure identification, but also high reproducibility, giving almost real-time identification results and possibilities to distinguish between bioagents, pathogenic and non-pathogenic species (Campos Braga et al., 2013). For example, MALDI-TOF MS was used to differentiate pathogenic and non-pathogenic *Leptospira* strains, by categorizing the super reference spectra and the major spectra projection dendrogram into groups, thus proving an important tool in surveillance of this omnipresent and zoonotic organism (Xiao et al., 2015). Furthermore, MALDI-TOF MS not only facilitates the detection of novel species, but can also reveal taxonomic anomalies (Spitaels et al., 2016; Wieme et al., 2014).

A new promising application of the method relates to non-culture identification of bacteria directly from samples containing mixed cultures (fluids, blood, urine), and protocols are being developed to improve the sensitivity of MS for that purpose (Opota et al., 2015), which is a great prospect for non-lethal health examination of farmed fish. Not all organisms in a mixed culture are identifiable, for example the analytical sensitivity in blood culture varied between 66 % and 76 %, with a dominant precision of about 90 % for identification of Gram-negative bacteria (Lavigne et al., 2013). Barnini et al. (2015) developed a method for MALDI-TOF MS rapid identification of Gram-negative bacteria and Gram-positive cocci from blood cultures, having higher precision and reliable results.

Aside from identification of bacteria, MALDI-TOF MS may be formulated for recognition of some crucial surface-associated virulence factors, based on the selective determination of presence or absence of *m/z* peaks (Bittar et al., 2009). A novel effector that contributes to virulence of the major causative agent of fish hemorrhagic septicemia and human pathogen, *Edwardsiella tarda*, was identified by MALDI-TOF MS (Xie et al., 2015). The most challenging application of MALDI-TOF MS is the ability to detect bacterial strains with developed antibiotic resistance (Kok et al., 2013). Different approaches were applied regarding particular antibiotics and microorganism classes. In Gram-negative bacteria, plasmid-encoded β-lactamases are the greatest contributors to antibiotic resistance, hydrolyzing the β-lactam structure of certain antibiotics, which leads to their inactivation (Kostrzewa et al., 2013). MALDI-TOF MS can be applied to measure enzyme-mediated, time-dependent hydrolysis of the β-lactam ring structure of penicillin G and ampicillin, as well as inhibition of hydrolysis by clavulanic acid (Hooff et al., 2012). When performed with growth media containing isotopically labeled amino acids, MALDI-TOF MS facilitates detection of resistant bacteria in less than three hours. Bacteria incorporate labeled amino acids, which increases their protein masses and shifts their peaks in spectral profile. In the presence of antibiotics, only resistant bacteria are able to grow and incorporate the labeled amino acids (Sparbier et al., 2013).

There is a number of evidences demonstrating reliability of MALDI-TOF MS when compared with other established methods, particularly 16S rRNA sequencing, and it is thus finding its way in environmental applications. As a rapid and inexpensive tool, it may serve for detection of bacteria for monitoring purposes and research, for health assessments and therapy considerations of aquatic organisms, as well as identification of bacterial contaminants and emerging indicator organisms that impact aquatic environments, acting as an early warning system for determination of causes of their impaired health.

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Table 1. Advantages and shortcomings of selected methods used for identification of environmental bacteria. Adapted from Buller (2004) and Okafor (2011).

Method	Advantages	Shortcomings
Biochemical identification kits (API systems, Vitek)	Easy to perform Requires limited training	Frequent misidentifications Limited profile index Requires cultivation of target organism and additional tests
Random amplification of polymorphic DNA (RAPD)	Rapid, easy to perform May be used to differentiate host source	Requires cultivation of target organism Libraries may be geographically and temporally specific
Amplified fragment length polymorphism (AFLP-PCR)	Highly reproducible May be used to differentiate host source	Requires specialized training Requires cultivation of target organism Requires reference library Libraries may be geographically and temporally specific Method varies according to different studies
Pulsed-field gel electrophoresis (PFGE)	Highly reproducible May be used to differentiate host source	Labor intensive, requires specialized training Requires cultivation of target organism Requires reference library Libraries may be geographically and temporally specific
Ribotyping	Highly reproducible Can be automated May be used to differentiate host source	Labor intensive, requires specialized training Requires cultivation of target organism Requires reference library Libraries may be geographically and temporally specific
Gene-specific PCR	Can be adapted to quantify gene copy number Virulence genes may be targeted Does not require reference library	Requires enrichment of target organism Sufficient quantity of target genes may not be available
Host-specific PCR	Rapid, easy to perform Does not require cultivation of target organism	Limited availability of primers
Real-time PCR	Rapid and reproducible In use for the microbial risk assessment of water quality	Requires high technical skill and support Requires costly reagents Not recommended for multiplex assays
16S ribosomal RNA sequencing	Rapid tool Best when used jointly with	Requires specialized training Less accurate at the species level

	biochemical tests	Not all primers bind to the DNA of the targeted organism
Fluorescence *in situ* hybridization (FISH)	Rapid, easy to perform Used increasingly for identification of bacteria from clinical samples	Requires cultivation of target organism Requires development of specific FISH assays for targeted organism Limited availability of primers

Table 2. Most frequently occurring bacterial pathogens of aquatic species. Bacteria are listed with respective names and symptoms of diseases, the tissues from which they can be retrieved, and aquatic animals where the organism has been reported. Adapted from Austin and Austin (1999), Buller (2004), Woo and Bruno (2011).

Pathogen/Identifiable from the MALDI-TOF MS databases	Disease	Signs of disease	Isolation tissue/host	Distribution
Aeromonas caviae (HG4)/yes	Septicemia, gastroenteritis, mortalities	Dermal ulcers, hepatopancreatic infections	Freshwater and ornamental fish, Atlantic salmon, giant freshwater prawn, turbot larvae, crayfish; Humans	Ubiquitous in the environment, worldwide
Aeromonas hydrophila ssp. *hydrophila* (HG1)/yes	Hemorrhagic septicemia, peritonitis, redsore disease, fin rot, red-fin disease, black disease, mortalities	Erosive or ulcerative dermal lesions, hemorrhage on fins and trunk, swelling of anus, erythema (fish); Hemorrhage on legs (frogs); Black nodules on thoracic appendages (crayfish)	Freshwater and ornamental fish, ayu, channel catfish, tilapia, trout, turtles, crayfish, reptiles, grey seal, occasionally marine fish and scallops, frogs, shrimps; Humans	Ubiquitous in the environment, worldwide
Aeromonas salmonicida ssp. *salmonicida* (HG3)/yes	Furunculosis of salmonid fish, goldfish ulcer disease	Dermal ulcers with typical furuncles; organisms penetrate to underlying tissues, kidney, spleen, intestine	Many species of freshwater and marine fish, frogs, crayfish	North America, Europe (highly virulent strain)
Aeromonas salmonicida ssp. *achromogenes*/yes	Variety of pathologies, carp erythrodermatitis, goldfish ulcer	Large open skin lesions surrounded by areas of	Organisms from freshwater, brackish water	Worldwide

	disease, ulcer disease of flounder, mortalities	descalation with hemorrhagic dermis	and marine environments	
Aeromonas veronii ssp. *sobria*/yes	Epizootic ulcerative fish syndrome, mortalities	Large ulcers all over the fish body, infectious dropsy, ascites	Freshwater and marine fish; Humans (the most pathogenic of all aeromonads)	Worldwide
Carnobacterium (*Lactobacillus*) *piscicola*/yes	Pseudokidney disease, lactobacillosis, post-stripping peritonitis	Visceral granulomas, epicarditis, peritonitis, ascites, blood or blisters under the skin	Salmonid fish, catfish	North America, Europe, Australia
Citrobacter freundii/yes	Systemic infections	Hemorrhagic spots on the skin, eye and fins.	Angelfish, trout, carp, crayfish, bullfrogs	In water, sewage, soil, worldwide
Edwardsiella ictaluri/yes	Enteric septicemia of catfish, chronic encephalitis	Petechial hemorrhage under jaw and belly; head, gill and kidney lesions, exophthalmia, gastroenteritis	Catfish, freshwater ornamental fish, bass, eels, crayfish, frogs, turtles	In water, sediment, organically polluted waters, Asia and North America
Edwardsiella tarda/yes	Edwardsiellosis, redpest, emphysematous putrefactive disease of catfish, fish gangrene	Septicemia, ulcerative dermatitis, intestinal infection, lesions in the muscle, kidney, liver	Many aquatic animals: fish, frogs, crayfish, amphibians, reptiles, mammals, turtles; Humans	Ubiquitous in the environment worldwide
Flavobacterium columnare (previously *Cytophaga columnaris, Flexibacter columnaris*)/yes	Columnaris disease, saddleback disease	Lesions on gills, ulcers and necrosis on body surface	Freshwater fish, crayfish	Worldwide

Hafnia alvei/yes	Hemorrhagic septicemia, mortalities	Swollen abdomen, furuncle-like lesions in kidney, darkening of skin	Salmonids, frogs, crayfish; Humans	Soil, sewage, water, Europe, Japan
Listonella anguillarum serotypes 01,02 (previously *Vibrio anguillarum*)/yes	Vibriosis, ulcerative disease, necrosis	Red spots on body surface, ulcerative skin lesions	Freshwater and marine fish, crayfish, molluscs	Worldwide
Mycobacterium spp./yes	Mycobacteriosis	Lesions on skin and kidneys, nodules in tissues	Freshwater and marine fish, crayfish, mollusks, frogs, reptiles, turtles, crocodiles: Humans	Worldwide
Pantoea (*Enterobacter*) *agglomerans*/yes	Hemorrhagic septicemia	Hemorrhages in eyes, dorsal musculature	Freshwater and marine fish; Humans	Worldwide
Photobacterium damselae ssp. *damselae* (previously *Vibrio damselae*, EF-5)/yes	Vibriosis, granulomatous ulcerative dermatitis	Skin ulcers, soft tissue infections	Freshwater and marine fish, mollusks, crayfish, turtles; Humans	Worldwide
Photobacterium damselae ssp. *piscicida* (previously *Pasteurella piscicida, Flavobacterium piscicida, Pseudomonas piscicida*)/yes	Pasteurellosis, fish pseudotuberculosis	White nodules in internal organs	Freshwater and marine fish	Worldwide, not in Australia
Renibacterium salmoninarum/yes	Bacterial kidney disease	Exophthalmia, blisters on flank, ulcers, abscesses, lesions over organs, necrotic abscesses in kidney	Salmonids	Worldwide, not in Australia
Tenacibaculum maritimum (previously	Columnaris disease, erosive skin disease, black	Eroded mouth and fins, ulcerated skin	Marine fish	North America, Europe, Asia

Cytophaga marina and *Flexibacter maritimus*)/yes	patch necrosis, mouth rot	lesions		
Vibrio fluvialis/yes	Mortalities	Lesions in internal organs	Mostly fish from brackish waters, marine mollusks, crustacea; Humans	Worldwide
Vibrio harveyi/yes	Vibriosis, mortalities in sharks and abalone	Necrotic degenerations, vacuolations in lesions, extended abdomen, red anus	Fish, mollusks, penaeid prawns and shrimps, octopus; Humans	Worldwide
Vibrio parahaemolyticus/yes	Withering syndrome, septicemia, mortalities	External hemorrhages, tail rot	Fish, crustaceans, abalone; Humans	Mostly Asia, spreading worldwide
Vibrio vulnificus biotype I/yes	Vibriosis, hemorrhagic septicemia, ulcerative disease	Ulcerous lesions, intestinal degenerations, necrosis	Marine and brackish fish, mollusks, crayfish, plankton; Humans	Worldwide
Yersinia ruckeri/yes	Enteric redmouth disease, yersiniosis	Reddening of throat and mouth, hemorrhages on gills, eyes, muscles	Freshwater and marine fish	Worldwide

Figure 1. Exemplary MALDI-TOF MS profiles of bacteria isolated from different freshwater crayfish tissues. Numbers refer to identification scores obtained with the Bruker MALDI-biotyper. Scores of 2.0 or higher are considered as highly probable species identification. Reprinted from Topić Popović et al., 2014.