Potential of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) for the identification of freshwater zooplankton: a pilot study with three *Eudiaptomus* (Copepoda: Diaptomidae) species

NICOLETTA RICCARDI[1], LARA LUCINI[2], CINZIA BENAGLI[2], MARTIN WELKER[3], BARBARA WICHT[2] AND MAURO TONOLLA[2,4]*

[1]CNR-INSTITUTE OF ECOSYSTEM STUDY, 28922 VERBANIA PALLANZA, ITALY, [2]CANTONAL INSTITUTE OF MICROBIOLOGY, VIA MIRASOLE 22A, CH-6500 BELLINZONA, SWITZERLAND, [3]ANAGNOSTEC GMBH, AM MÜHLENBERG 11, 14476 POTSDAM-GOLM, GERMANY AND [4]MICROBIAL ECOLOGY, MICROBIOLOGY UNIT, BIVEG DEPARTMENT, UNIVERSITY OF GENEVA, 30, QUAI ERNEST ANSERMET, 3000 GENEVA, SWITZERLAND

*CORRESPONDING AUTHOR: mauro.tonolla@unige.ch

The accurate identification of individuals in zooplankton samples is a crucial step in many plankton studies. Up to now, this has been done primarily by microscopic analysis of morphological characters, and new molecular methodologies are still relatively rarely applied. Another promising technology is matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), which has had a major impact in applied and systematic microbiology, where it is used for routine high throughput identification of bacteria and fungi. For the present study, we developed a protocol for the rapid acquisition of mass spectra from whole individual copepods. The final protocol enabled us to obtain mass spectra with more than 100 distinct peaks in the mass range of 2000−20 000 Da. A comparison of the mass spectra of three species of *Eudiaptomus* showed that they could all be clearly discriminated, whereas the mass spectra of different developmental stages and sexes of each particular species were highly similar. Further, a discrimination of con-specific individuals from different habitats was achieved, at least partly, even without extensive optimization of the analytical and statistical procedures. These results indicate the feasibility of identifying copepods by a rapid and simple MALDI-TOF MS analysis, e.g. for population ecology studies.

KEYWORDS: *Eudiaptomus*; MALDI-TOF MS; identification

INTRODUCTION

The taxonomic discrimination of zooplankton species relies on subtle morphological characteristics, which makes routine identification of individuals very difficult and laborious, and requires considerable experience (Mauchline, 1998; Thum and Derry, 2008; Jagadeesan *et al.*, 2009). For example, copepod species identification generally involves careful dissection and examination of

minute morphological characters located on different parts of the male or the female, such as the female genital somite and the male mating appendages in calanoid families/genera (Ranga Reddy, 1994; Dussart and Defaye, 1995), and/or the fourth leg coxa and the fifth pair of legs in cyclopoids and poecilostomatoids (Einsle, 1996; Karaytug, 1999; Böttger-Schnack and Schnack, 2009). Depending on which morphological feature proves most useful for species discrimination, taxonomic keys to species within each genus are frequently based on either male or female characters, while equally detailed descriptions of both sexes are rarely provided (Mauchline, 1998). For instance, taxonomic keys for the identification of *Eudiaptomus* species are generally based on the morphology of males due to the lack of unambiguous morphological differences between females (Kiefer, 1968; Petkowski, 1983; Stella, 1984). This contributes to a recurring problem in routine zooplankton analysis, matching the females to the males of each species. Moreover, immature copepod stages cannot generally be identified to species (or even genus) level (MacManus and Katz, 2009; Bucklin *et al.*, 2010) and are pooled under more or less generic categories that can even include the total number of copepod nauplii or copepodites in multi-species copepod assemblages.

Studies of zooplankton assemblages are therefore often limited by the impossibility of assessing the actual abundance of each species population, which affects biodiversity and ecological assessment. This applies to all groups of holozooplankton, including calanoid copepods, for which species identification is further complicated by the existence of several groups of closely related sibling species that can be difficult or impossible to distinguish using morphological characters (Thum and Derry, 2008). Since accurate taxonomic species identification at all life stages is critical for ecological studies, the use of molecular methods complementary to traditional morphological analysis is rapidly growing (MacManus and Katz, 2009). Molecular approaches, including DNA barcoding and community metagenomics, can assess species diversity (Bucklin *et al.*, 2010; Radulovici *et al.*, 2010), reveal cryptic and new species (Belyaeva and Taylor, 2009), elucidate phylogenetic relationships (Bucklin and Frost, 2009) and identify species from diapausing eggs (Briski *et al.*, 2011). However, most of these methods are applied in ecological studies with a focus on evolutionary questions (Selkoe and Toonen, 2006; Costa and Carvalho, 2010; Yebra *et al.*, 2011). For the rapid identification of individuals, techniques based on nucleotide sequences (still) have the disadvantage of requiring a number of steps, i.e. DNA extraction, polymerase chain reaction, gel electrophoresis, etc. Another approach for fast, high throughput identification of zooplankton specimens could be the use of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), a technology that has had a major impact in many fields of the life sciences over the last two decades (Duncan *et al.*, 2008; Karr, 2008; Seng *et al.*, 2010; Welker, 2011).

MALDI-TOF MS of whole cells has been recently applied to the identification of bacteria and fungi in clinical, plant and veterinary microbiology (Seng *et al.*, 2009; Kallow *et al.*, 2010, Rezzonico *et al.*, 2010). This technology takes advantage of the fact that whole cells can be analyzed after a simple single-step extraction procedure without any further preparation steps. The resulting mass spectra display peak patterns that have been shown to be phylogenetic markers in a mass range of $2-20$ kDa (Wynne *et al.*, 2009). For bacteria, or prokaryotes in general, it has been shown that the peaks recorded in MALDI-TOF mass spectra can be assigned to ribosomal proteins (Teramoto, 2009; Kallow *et al.*, 2010), thus representing biomarkers that can be used in taxonomic studies (Kroppenstedt *et al.*, 2005) and also for routine identification (Freiwald and Sauer, 2009).

Among eukaryotes, it is primarily yeasts and moulds of importance in medical and food safety that have been analyzed by MALDI-TOF MS (Erhard *et al.*, 2008; Marinach-Patrice *et al.*, 2009). An equal potential for species discrimination and identification has been recognized, although the identity of the compounds detected (mostly proteins) is not fully understood: along with ribosomal proteins, it is mostly cell-wall-associated proteins that seem to be recorded (Hettick *et al.*, 2008).

The excellent results obtained with microorganisms have prompted a number of studies to assess the potential of MALDI-TOF MS for discriminating species of metazoa. The outcome of these studies is equally promising, as they have shown that species discrimination based on mass spectral pattern is possible for nematodes (Perera *et al.*, 2005a), various insect taxa (Perera *et al.*, 2005b; Feltens *et al.*, 2010; Kaufmann *et al.*, 2011), bivalves (Lopez *et al.*, 2005) and fish (Mazzeo *et al.*, 2008; Volta *et al.*, 2012).

In this context, MALDI-TOF MS analysis appears to be a promising tool for plankton ecology studies. Its potential in limnological studies has already been shown, for example, by the clonal typing of *Microcystis* (Cyanobacteria) colonies based on metabolomic profiles (Welker *et al.*, 2007). In the present pilot study, we sought to explore the potential of mass spectral analysis for the taxonomic identification of zooplankton. We chose diaptomid copepods (Copepoda: Calanoida: Diaptomidae), the most diverse group of freshwater calanoids, with over 400 species in more than 50 genera (for a complete list of species, see http://www

.nmnh.si.edu/iz/copepod). We focused on three of the five species of *Eudiaptomus* recorded in Italian waters (Stoch, 2005) which are most representative of the calanoid assemblages of the Northern Italian water bodies (Stella, 1984; Tavernini *et al.*, 2003; Riccardi and Rossetti, 2007).

The aims of the study were, first, to develop a simple and straightforward protocol for mass spectral analysis of individual copepods, and secondly, to evaluate this technology in discriminating the three species as a prerequisite for the MALDI-TOF MS-based identification of these organisms.

METHOD

Sample collection and specimen identification

Eudiaptomus populations were collected with a 126 μm plankton net from six lakes and one artificial basin (Table I) between April and July 2009 and preserved in 95% ethanol until analysis. The specimens were not preserved in formalin because microbiological studies have demonstrated that formalin affects the acquisition of high-quality mass spectra due to protein denaturation and degradation. *Eudiaptomus* specimens were identified using the taxonomic literature of Dussart (Dussart, 1967), Stella (Stella, 1984), Kiefer (Kiefer, 1968, 1978), Petkowski (Petkowski, 1983) and Einsle (Einsle, 1993). Reference specimens were examined for morphological differences, dissected in glycerine and stored in sealed slides. Species identification was made by examining the geniculate right antennule and the modified fifth legs of males, the shape of the genital somite and the morphology of the lateral wings of the fifth pediger and the fifth legs of females.

For mass spectral analysis, adults of both sexes were individually sorted from each sample under a dissecting microscope. Only individuals apparently free from epibionts and parasites were used for analysis. Because the copepodite stages of *Eudiaptomus gracilis* and *Eudiaptomus*

padanus are almost indistinguishable, we considered only copepodites of the third *Eudiaptomus* species (*Eudiaptomus intermedius*), which never co-occur with the other two species (Tavernini *et al.*, 2003). Males, females and copepodites of *Mixodiaptomus tatricus* were obtained from different collections, analyzed and used as an outgroup.

Mass spectral analysis

The first part of the study involved optimizing the sample preparation procedure and the settings for data acquisition by MALDI-TOF MS. One hundred and two (102) specimens were used to compare the effects of sample preparation on analytical output and to define a protocol that was then applied to a further 122 specimens, which were analyzed and the data evaluated.

Three different matrices were tested: 2,4-dihydroxybenzoic acid (DHB), sinapinic acid (SA) and alpha-cyano-4-hydroxycinnamic acid (CHCA). Solid matrix compounds (75 mg mL^{-1} DHB, 40 mg mL^{-1} SA and 40 mg mL^{-1} CHCA, respectively) were dissolved in a mixture of acetonitrile, ethanol 96% and distilled water (30% each) acidified with 3% v/v trifluoroacetic acid.

We tested two protocols for protein extraction from whole individuals. In the first, individuals were placed in a reaction tube in 5 μL of matrix solution, and extraction was performed passively or by sonification for 5, 10, 15 and 30 s in a water bath. Of the resulting extract, 0.5 μL was deposited on a stainless steel target plate (FlexiMass, Kratos, Manchester, UK). In the second protocol, individual copepods were placed directly on the target plate and proteins were directly extracted on each sample spot by adding 0.5 μL of matrix solution.

Although the analysis of samples prepared according to the second protocol gave reproducible results with acceptable quality of the mass spectra, this approach was not followed up due to the risk of contaminating the vacuum chamber by sample particles coming from the target plate during measurement. The extraction procedure chosen was to submerge one single copepod in

Table I: Sampling sites of Eudiaptomus *and* Mixodiaptomus *species analyzed*

Sampling site	Geographic location	Habitat type	Altitude (m a.s.l.)	Species
Idroscalo Milan	45°27′42″N 9°17′18″E	Artificial pond, eutrophic,	122	*E. gracilis*
Lake Maggiore	45°56′0″N 8°32′0″E	Large, meso-oligotrophic,	193	*E. gracilis*
Lake Santo	44°24′11″N 10°00′22″E	Small, oligotrophic	1507	*E. intermedius*
Lake Scuro	44°28′31″N 10°5′28″E	Small, oligotrophic	1526	*E. intermedius*
Lake Monate	45°47′57″N 8°39′34″E	Small, meso-oligotrophic	266	*E. padanus*
Lake Mergozzo	45°57′21″N 8°27′56″E	Small, oligotrophic	204	*E. padanus*
Lake Comabbio	45°45′53″N 8°41′24″E	Shallow, hypereutrophic	243	*E. padanus*
Sennes pond	46°39′36″N 12°3′0″E	Small, shallow, oligotrophic	2120	*M. tatricus*

5 μL of the CHCA matrix solution for 10 min. During this time, the soft tissues and chitinous carapace dissolved totally or partly. Sonication to enhance cell lysis and extraction did not lead to a significant improvement in peak intensity and peak number in mass spectra and was thus omitted.

A volume of 0.5 μL of the crude extract obtained was spotted in quadruplicates on the target plate and allowed to evaporate for 1−2 min at room temperature. Crystal formation on the sample position was controlled visually.

Protein mass fingerprints (Kallow *et al.*, 2010) were obtained using an AXIMA^{TM} Confidence instrument (Shimadzu-Biotech Corp., Kyoto, Japan), with spectrum acquisition in a linear, positive ion extraction mode at a laser frequency of 50 Hz and within a mass range of 2000−20 000 Da. The acceleration voltage was 20 kV, and the extraction delay time was 200 ns. Protein mass fingerprints were accumulated from 100 profiles, each in turn being accumulated from five laser pulse cycles with the auto-quality option turned on in the instrument's software. The resulting raw spectra were thus accumulated from 500 laser pulse cycles and processed using the Launchpad^{TM} v. 2.8 software (Shimadzu-Biotech Corp.) by smoothing with a filtering width of 50 channels and baseline subtraction with a filter width of 500 channels. Peak detection was performed with the threshold-apex peak detection method, applying the adaptive voltage threshold which follows the signal noise level. For each raw mass spectrum, a peak list was generated that included for each peak the apex m/z value, the mass deviations and the signal intensity. Calibration was performed for each target plate using the reference strain *Escherichia coli* K12 (GM48 genotype).

Data analysis and handling

Generated protein mass fingerprints (peak lists) were imported into the SARAMIS^{TM} software (Spectral Archive and Microbial Identification System, AnagnosTec GmbH) and analyzed using the following pre-setting parameters: mass range from 2000 to 20 000 Da, allowed mass deviation of 800 ppm. With these settings, a set of so-called supermasses was extracted from mass spectra of specimens belonging to the same species. These are mass signals that have been recorded, with the permitted analytical error range, with a frequency of at least 80%. The resulting pattern of supermasses represents a consensus mass spectra pattern for individual species.

The individual mass spectra were further analyzed by cluster analysis, applying a single link agglomerative clustering algorithm in SARAMIS^{TM}. The clustering was based on the absence or presence of peaks and not on their intensities.

RESULTS

For the present study, we focused on three species of *Eudiaptomus* which are the most widely distributed in Northern Italian lakes: (i) *E. intermedius*, an Eastern alpine—Illyric species (Stella, 1979), (ii) *E. padanus* (subspecies *E. padanus padanus*), one of the most common calanoids in Italian prealpine water bodies (Stella, 1984), and (iii) *E. gracilis*, which is broadly distributed throughout different continents (Dussart and Defaye, 2002) but which has started expanding in Northern Italy only recently (Riccardi and Rossetti, 2007). Copepod populations were sampled from water bodies representative of a wide range of environmental conditions (Table I). *Eudiaptomus intermedius*, a species typical of permanent water bodies in the Northern Apennines (Tavernini *et al.*, 2003), was obtained from two oligotrophic lakes where other diaptomid species are not present. *Eudiaptomus padanus*, an endemic species which is gradually being displaced by *E. gracilis* (Riccardi and Rossetti, 2007), was obtained from three lakes of different trophic degree, where it is still the only diaptomid present. The third species, *E. gracilis*, was the only diaptomid copepod in the eutrophic artificial Idroscalo reservoir, while it co-occurred with rare *E. padanus* individuals in the large, meso-oligotrophic Lake Maggiore. The sample of *M. tatricus* was collected from a high-altitude pond in the Alpine protected area of Fanes-Sennes-Braies (Dolomites), which sustains only one calanoid species (Marrone and Stoch, unpublished data).

This first approach to a MALDI-TOF MS-based identification of copepods involved comparing different matrices for sample deposition before the automated spectra acquisition procedure. Of the three different matrices tested (DHB, SA and CHCA), the last was chosen because it yielded the most reproducible results, most probably due to the homogeneous distribution of sample/matrix crystals on the target plate. The two other matrix compounds crystallize in larger needles, which form a heterogeneous distribution on the sample position, requiring a higher number of laser pulse cycles to yield a representative average mass spectrum (data not shown).

MALDI-TOF MS analysis of crude extracts of whole *Eudiaptomus* individuals using CHCA revealed complex mass spectral patterns in the m/z range of 2000−20 000 Da for all the species analyzed. Most
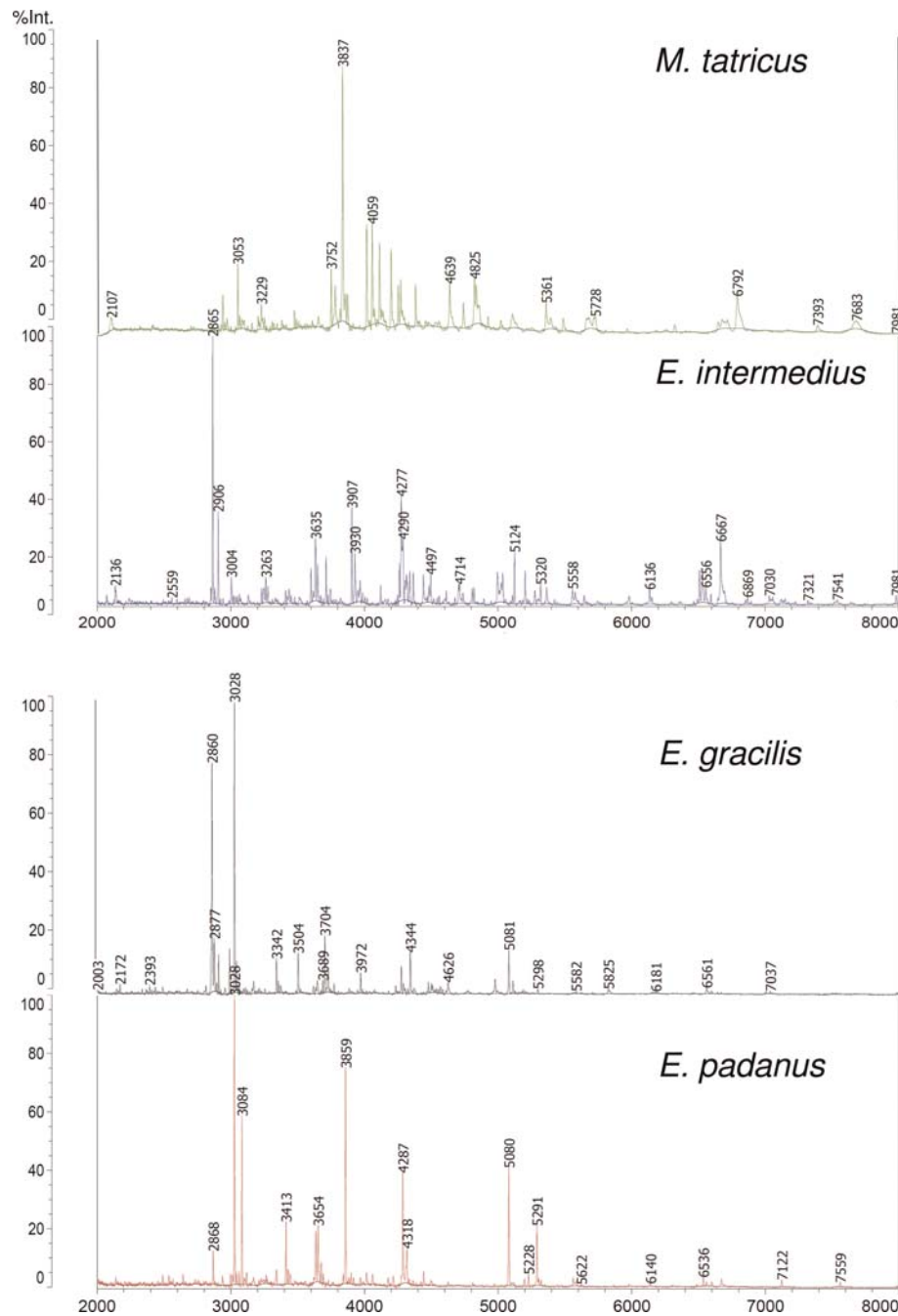
Fig. 1. Comparison of protein mass spectra of organisms belonging to *E. gracilis*, *E. intermedius, E. padanus* and *M. tatricus*. Similar and diverging marker masses characteristic of these *Eudiaptomus* species are listed in Supplementary data, Table SI.

peaks of high intensity were recorded in the mass range between 2 and 6 kDa.

Quadruplicates of the samples yielded reproducible patterns from sample position to sample position, also from different target plates. Figure 1 shows typical spectral patterns of proteins extracted from Eudiaptomus and Mixodiaptomus specimens obtained in the automated spectrum acquisition mode. Generally speaking, a number of some 10–15 peaks with a relative intensity exceeding 10% was recorded, along with some 100 further peaks of intermediate (1–10%) or low (<1%) intensity. This result is similar to those for mass spectral patterns of whole bacterial cells. The peak lists extracted from the raw spectra were taken for further data

analysis, as provided by the Launchpad software. In consequence, replicate mass spectra of individual copepods were not identical, which was, however, not regarded as a major problem, as this is a well-known occurrence in the analysis of microorganisms. Our reasons for using this analysis to assess variability are intrinsic to MALDI-TOF MS itself: heterogeneities occur during co-crystallization of matrix and analytes, and during ionization, they are not leveled out with a finite number of laser pulse cycles (Dreisewerd, 2003). As a consequence, it is primarily peaks of low intensity close to the threshold that cause analysis-to-analysis variability, while peaks of intermediate and high intensity were found to be generally stable.

Tentative protein assignment to individual peaks, using *in silico* database searches, e.g. the Tag-Ident proteomics tool or the ExPASy Sequence Retrieval System (http://us.expasy.org), was not possible due to the lack of genomic and proteomic data, not only for species of the genus *Eudiaptomus* but also for the entire Copepoda order. The peak patterns were therefore taken as biomarkers as such without further identification of the identities of individual peaks.

A comparison of spectral fingerprints of individuals belonging to *E. gracilis*, *E. intermedius* and *E. padanus* revealed species-specific differences in the peak patterns (Fig. 1). The mass spectra of the three *Eudiaptomus* species were distinctly different from those of *M. tatricus*, which produced a clearly separated clade in the dendrogam (Fig. 2).

Subsets of peaks characteristic of each species are shown in Supplementary data, Table SI. These peaks represent the *m/z* values of a set of prominent peaks detected in at least 80% of the individual mass spectra considered. A number of peaks were recorded for two or three species, while others appear to be unique for a single species. Such patterns of variably shared peaks have also been observed in studies on closely related bacterial species, where they are interpreted as variations in homologous proteins due to amino acid exchanges caused by point mutations (Dieckmann *et al.*, 2010).

Among the females, males and copepodites of individual species, differences between peak patterns were generally lower than between species (Fig. 2). This indicates that differential protein expression patterns of developmental stages and sexes are only moderate and contribute to a lower degree of heterogeneity than the analytical variability itself. Thus, MALDI-TOF MS peak patterns are arguably stable mass fingerprints of individual taxonomic units, and can be applied to taxonomic identification independently of sex and developmental stage.

In the dendrogram computed from the mass fingerprints, the three species considered were clearly separated, irrespective of the origin of the samples (Fig. 2). Nevertheless, subclusters including individuals from a specific lake appeared for most species. This was found to be most pronounced for *E. intermedius*, with
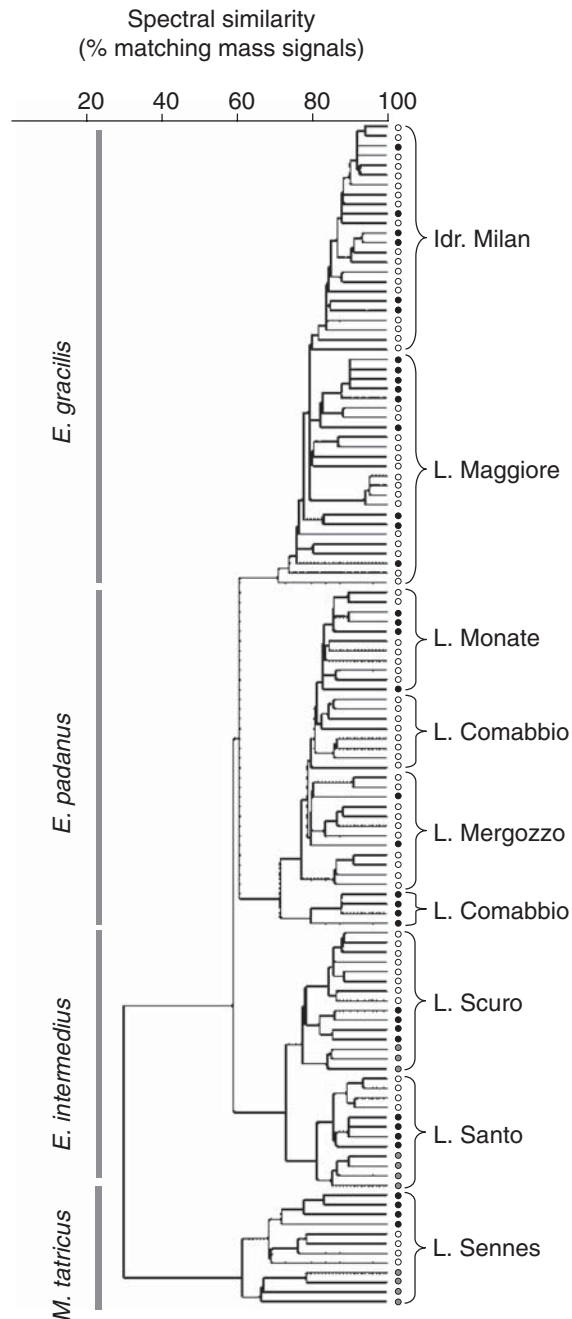

Fig. 2. Dendrogram based on the protein mass fingerprint patterns of all analyzed organisms (females, empty circle; males, full circle; copepodids, gray circle) using the single-link clustering algorithm implemented in SARAMIS.

individuals of populations from L. Scuro and L. Santo clearly separated in distinct clusters. For *E. gracilis*, only individuals from the Milan Idroscalo were grouped in a distinct cluster, while those from L. Maggiore appear to be more diverse, with smaller distinct subclusters. Lastly, individuals of *E. padanus* were grouped in several smaller subclusters not fully consistent with the individuals' origin.

DISCUSSION

It must be emphasized that the results presented here are only a pilot study.

Nevertheless, clear differentiation between species was achieved, although all the settings and statistical algorithms used were originally designed for the analysis and identification of bacteria and fungi. This indicates that even such preliminary results as those presented here are contributing to the growing evidence of the reliability of MALDI-TOF-based identification of multicellular organisms. Following promising pioneer work on nematodes and insects (Campbell, 2005; Perera, 2005a, b), MALDI-TOF protein profiling is currently applied to the identification of insect (e.g. Kaufmann *et al.*, 2011) and fish species (Mazzeo *et al.*, 2008; Volta *et al.*, 2012). Our study appears to be the first application to crustacean zooplankton (i.e. copepods), as well as the first to use field-collected whole organisms for analysis. It is true that only wild specimens were used for the studies on fish, but the analyses were performed on muscle tissue samples. In contrast, laboratory-reared strains were analyzed for the insect studies referred to above. This probably resulted in a reduction in environmental-induced variations, a potential source of individual or between-strain variability and thus of an increase in the probability of inconsistent results (Campbell, 2005), which was expected to be a major concern in the application of this technique to the identification of complex multicellular organisms. Potential sources of inconsistency might include sex, age and/or stage of development and all the differences in individual diet and/or hosted biota (commensal or parasitic organisms). In fact, a considerable impact of the stomach blood content on the MALDI-TOF pattern of the haematophagous female midge *Culicoides nubeculosus* was observed (Kaufmann *et al.*, 2011), while sex or age had negligible effects on the mass spectra of the fruit flies *Drosophila melanogaster* (Campbell, 2005) and *C. nubeculosus* (Kaufmann *et al.*, 2011). Accordingly, we did not observe sex or age effects on the mass spectra of *Eudiaptomus* and *Mixodiaptomus* species. The above-mentioned studies on insects led to the conclusion that

the MALDI-TOF-based method is likely to be robust in the face of such variations, and can be used for the unambiguous identification even of very closely related species. Our results, though preliminary, seem to support this hypothesis. Indeed, even though we analyzed wild specimens that were probably carrying different periphyton and had a different gut content, differences between individual mass spectra were not so large as to influence species discrimination. This suggests that an optimization of both mass spectral analysis and data processing for this particular type of sample is worth performing to exhaustively evaluate the potential of MALDI-TOF MS analysis for plankton studies. For instance, a detailed analysis of mass fingerprints may reveal biomarkers that can distinguish subpopulations or reveal cryptic species (e.g. Campbell, 2005). An indication of this kind of potential seems to emerge from the subclustering distribution of the individuals within each of the *Eudiaptomus* species analyzed. However, testing this hypothesis demands first of all an accurate evaluation of the influence of the potential sources of "contamination" (e.g. gut content, endo- and ecto-parasites and commensalists) and of individual physiological status on mass spectra. For instance, spectra reproducibility within a population could be altered if larval stages (e.g. nauplii) are compared with post-metamorphic stages (copepodites). The extreme simplicity of the MALDI-TOF technique aids in the rapid performance of such comparisons. In fact, the high degree of automation of the analytical procedure means that several hundred individuals can be prepared and measured per day, allowing the analysis of populations in statistically required numbers. In the present study, mass spectral analysis has not reached its limit of detection, i.e. it should be feasible to analyze even smaller samples. Further studies will therefore include the possibility of analyzing nauplii and/or eggs by miniaturizing the sample preparation procedure.

This study suggests that MALDI-TOF MS analysis can provide an alternative or complementary tool for species identification and recognition in plankton research. While methods based on molecular biology generally demand some *a priori* knowledge of the sample under investigation (e.g. for primer selection) and, more importantly, can be slow and laborious with results that may depend on experimental variables (e.g. DNA extraction, temperature-sensitive restriction), a mass spectral approach would be much more straightforward, requiring minimal time and relatively low costs. At the same time, it would be an accurate and sensitive tool not requiring any previous information of the samples to be analyzed, as is the case for all kinds of microorganisms. A prerequisite is the creation of a

taxonomically comprehensive molecular profile database for the known zooplankton species, to allow rapid and accurate identification of specimens and provide faster and more detailed characterization of species distribution and diversity patterns.

SUPPLEMENTARY DATA

Supplementary data can be found online at http:// plankt.oxfordjournals.org.

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REFERENCES

Belyaeva, M. and Taylor, D. J. (2009) Cryptic species within the *Chydorus sphaericus* species complex (Crustacea: Cladocera) revealed by molecular markers and sexual stage morphology. *Mol. Phylogenet. Evol.*, **50**, 534–546.

Böttger-Schnack, R. and Schnack, D. (2009) Taxonomic diversity and identification problems of oncaeid microcopepods in the Mediterranean Sea. *Mar. Biodivers.*, **39**, 131–145.

Briski, E., Cristescu, M. E., Bailey, S. A. *et al.* (2011) Use of DNA barcoding to detect invertebrate invasive species from diapausing eggs. *Biol. Invasions*, **13**, 1325–1340.

Bucklin, A. and Frost, B. W. (2009) Morphological and molecular phylogenetic analysis of evolutionary lineages within *Clausocalanus* (Crustacea, Copepoda, Calanoida). *J. Crust. Biol.*, **29**, 111–120.

Bucklin, A., Hopcroft, R. R., Kosobokova, K. N. *et al.* (2010) DNA barcoding of Arctic Ocean holozooplankton for species identification and recognition. *Deep-Sea Res. II*, **57**, 40–48.

Costa, F. O. and Carvalho, G. R. (2010) New insights into molecular evolution: prospects from the Barcode of Life Initiative (BOLI). *Theory Biosci.*, **129**, 149–157.

Dieckmann, R., Strauch, E. and Alter, T. (2010) Rapid identification and characterization of *Vibrio* species using whole-cell MALDI-TOF mass spectrometry. *J. Appl. Microbiol.*, **109**, 199–211.

Dreisewerd, K. (2003) The desorption process in MALDI. *Chem. Rev.*, **103**, 395–425.

Duncan, M. W., Roder, H. and Hunsucker, S. W. (2008) Quantitative matrix-assisted laser desorption/ionization mass spectrometry. *Brief. Funct. Genom. Proteom.*, **7**, 355–370.

Dussart, B. (1967) *Les Copépodes des eaux continentales d'Europe Occidentale. I. Calanoïdes et Harpacticoïdes*. Boubée et Cie, Paris, France.

Dussart, B. and Defaye, D. (2002) *World Directory of Crustacea Copepoda of Inland Waters. I—Calaniformes*. Backuhys, Kerkwerve, Netherlands.

Dussart, B. H. and Defaye, D. (1995) Introduction to the Copepoda. In Dumont, H. J. F. (ed.), *Guides to the Identification of the Microinvertebrates of the Continental Waters of the World*. Vol. 7. SPB Academic Publishing, Amsterdam.

Einsle, U. (1993) *Crustacea Copepoda Calanoida und Cyclopoida. Süβwässer Fauna von Mitteleuropa*. 8/4. Gustav Fischer Verlag, Jena, Germany.

Einsle, U. (1996) Copepoda: Cyclopoida. Genera *Cyclops, Megacyclops, Acanthocyclops*. In Dumont, H. J. F. (ed.), *Guides to the Identification of the Microinvertebrates of the Continental Waters of the World*. Vol. 7. SPB Academic Publishing, Amsterdam.

Erhard, M., Hipler, U. C., Burmester, A. *et al.* (2008) Identification of dermatophyte species causing onychomycosis and tinea pedis by MALDI-TOF mass spectrometry. *Exp. Dermatol.*, **17**, 365–371.

Feltens, R., Görner, R., Kalkhof, S. *et al.* (2010) Discrimination of different species from the genus *Drosophila* by intact protein profiling using matrix-assisted laser desorption ionization mass spectrometry. *BMC Evol. Biol.*, **10**, 95.

Freiwald, A. and Sauer, S. (2009) Phylogenetic classification and identification of bacteria by mass spectrometry. *Nat. Protoc.*, **4**, 732–742.

Hettick, J. M., Green, B. J., Buskirk, A. D. *et al.* (2008) Discrimination of *Aspergillus* isolates at the species and strain level by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry fingerprinting. *Anal. Biochem.*, **380**, 276–281.

Jagadeesan, L., Perumal, P. and Thangaraj, L. (2009) Molecular identification of marine calanoid copepod *Paracalanus parvus*. *World J. Fish Mar. Sci.*, **1**, 239–242.

Kallow, W., Erhard, M., Shah, H. N. *et al.* (2010) MALDI-TOF MS and microbial identification: years of experimental development to an established protocol. In Shah, H. N., Gharbia, S. E. and Encheva, V. (eds), *Mass Spectrometry for Microbial Proteomics*. Wiley, Chichester, UK, pp. 255–276.

Karaytug, S. (1999) Copepoda: Cyclopoida. Genera Paracyclops, Ochridacyclops and key to the Eucyclopinae. In Dumont, H. J. F. (ed.), *Guides to the Identification of the Microinvertebrates of the Continental Waters of the World*. Vol. 7. SPB Academic Publishing, Amsterdam.

Karr, T. L. (2008) Application of proteomics to ecology and population biology. *Heredity*, **100**, 200–206.

Kaufmann, C., Ziegler, D., Schaffner, F. *et al.* (2011) Evaluation of matrix-assisted laser desorption/ionization time of flight mass spectrometry fro characterization of *Culicoides nubeculosus* biting midges. *Med. Vet. Entomol.*, **25**, 32–38.

Kiefer, F. (1968) Versuch einer Revision der Gattung *Eudiaptomus* Kiefer (Copepoda Calanoida). *Mem. Ist. Ital. Idrobiol.*, **24**, 9–160.

Kiefer, F. (1978) *Das Zooplankton der Binnengewässer. Freilebende Copepoda. Die Binnengewässer.* Band 26. Teil 2. E., Schweizerbart'sche Verlag, Stuttgart, Germany.

Kroppenstedt, R. M., Mayilraj, S., Wink, J. M. *et al.* (2005) Eight new species of the genus *Micromonospora, Micromonospora citrea* sp. nov., *Micromonospora echinaurantiaca* sp. nov., *Micromonospora echinofusca* sp. nov., *Micromonospora fulviviridis* sp. nov., *Micromonospora inyonensis* sp. nov., *Micromonospora peucetia* sp. nov., *Micromonospora sagamiensis* sp. nov., and *Micromonospora viridifasciens* sp. nov. *Syst. Appl. Microbiol.*, **28**, 328–339.

Lopez, J. L., Abalde, S. L. and Fuentes, J. (2005) Proteomic approach to probe for larval proteins of the mussel *Mytilus galloprovincialis*. *Mar. Biotechnol. (NY)*, **7**, 396–404.

MacManus, G. B. and Katz, L. A. (2009) Molecular and morphological methods for identifying plankton: what makes a successful marriage? *J. Plankton Res.*, **31**, 1119–1129.

Marinach-Patrice, C., Lethuillier, A., Marly, A. *et al.* (2009) Use of mass spectrometry to identify clinical *Fusarium* isolates. *Clin. Microbiol. Infect.*, **15**, 634–642.

Mauchline, J. (1998) *The Biology of Calanoid Copepods. Advances in Marine Biology*, Vol. 33. Academic Press, London.

Mazzeo, M. F., Giulio, B. D., Guerriero, G. *et al.* (2008) Fish authentication by MALDI-TOF mass spectrometry. *J. Agric. Food Chem.*, **56**, 11071–11076.

Perera, M. R., Vanstone, V. A. and Jones, M. G. K. (2005a) A novel approach to identify plant parasitic nematodes using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom.*, **19**, 1454–1460.

Perera, M. R., Vargas, R. D. F. and Jones, M. G. K. (2005b) Identification of aphid species using protein profiling and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Entomol. Exp. Appl.*, **117**, 243–247.

Petkowski, T. K. (1983) *Calanoïdes—Calanoida (Crustacea—Copepoda). Faune de Macedoine.* V. Museé Histoire Naturelle de la Macedoine, Skopje.

Radulovici, A. E., Archambault, P. and Dufresne, F. (2010). DNA barcodes for marine biodiversity: moving fast forward? *Diversity*, **2**, 450–472.

Ranga Reddy, Y. (1994) Copepoda: Calanoida: Diaptomidae. Key to the genera *Heliodiaptomus, Allodiaptomus, Neodiaptomus, Phyllodiaptomus, Eodiaptomus, Arctodiaptomus and Sinodiaptomus*. In Dumont, H. J. F. (ed.), *Guides to the Identification of the Microinvertebrates of the Continental Waters of the World*. Vol. 7. SPB Academic Publishing, Amsterdam.

Rezzonico, F., Vogel, G., Duffy, B. *et al.* (2010) Application of whole-cell matrix-assisted laser desorption ionization-time of flight mass spectrometry for rapid identification and clustering analysis of Pantoea species. *Appl. Environ. Microbiol.*, **76**, 4497–4509.

Riccardi, N. and Rossetti, G. (2007) *Eudiaptomus gracilis* in Italy: how, where and why. *J. Limnol.*, **66**, 64–69.

Selkoe, K. A. and Toonen, R. J. (2006) Microsatellites for ecologists: a practical guide to using and evaluating microsattelite markers. *Ecol. Lett.*, **9**, 615–629.

Seng, P., Drancourt, M., Gouriet, F. *et al.* (2009) Ongoing revolution in bacteriology: routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *Clin. Infect. Dis.*, **49**, 543–551.

Seng, P., Rolain, J. M., Fournier, P. E. *et al.* (2010) MALDI-TOF mass spectrometry applications in clinical microbiology. *Future Microbiol.*, **5**, 1733–1754.

Stella, E. (1979) Considerazioni biogeografiche sui Diaptomidi (Copepoda Calanoida) delle acque dolci italiane. *Lav. Soc. Ital. Biogeo.*, **6**, 315–328.

Stella, E. (1984) *Crustacea—Copepoda: Calanoida d'acqua dolce.* Calderini, Bologna.

Stoch, F. (2005) Crustacea Copepoda Calanoida. In Ruffo, S. and Stoch, F. (eds), *Checklist e distribuzione della fauna italiana. Memorie del Museo Civico di Storia Naturale di Verona*, 2. serie, Sezione Scienze della Vita, pp. 91–92.

Tavernini, S., Fratta, E., Sartore, F. *et al.* (2003). Distribution and ecology of calanoid species in relation to morphometric and chemical characteristics of lakes and ponds of the Northern Appennines (Italy). *J. Limnol.*, **62**, 28–34.

Teramoto, K. (2009) Rapid identification and classification of bacteria using ribosomal protein as biomarkers by matrix-assisted laser desorption/ionization-mass spectrometry. *Bunseki Kagaku*, **58**, 971–972.

Thum, R. A. and Derry, A. M. (2008) Taxonomic implications for diaptomid copepods based on contrasting patterns of mitochondrial DNA sequence divergences in four morphospecies. *Hydrobiologia*, **614**, 197–207.

Volta, P., Riccardi, N., Lauceri, R. *et al.* (2012) Characterization and separation of freshwater fish species by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS): a pilot study. *J. Limnol.*, **71**, doi: 10.4081/jlimnol.2012.e17.

Welker, M. (2011) Proteomics for routine identification of microorganisms. *Proteomics*, **11**, 1–11.

Welker, M., Sejnohova, L., von Döhren, H. *et al.* (2007) Seasonal shifts in chemotype composition of *Microcystis* sp. communities in the pelagial and the sediment of a shallow reservoir. *Limnol. Oceanogr.*, **52**, 609–619.

Wynne, C., Fenselau, C., Demirev, P. A. *et al.* (2009) Top-down identification of protein biomarkers in bacteria with unsequenced genomes. *Anal. Chem.*, **81**, 9633–9642.

Yebra, L., Bonnet, D., Harris, R. P. *et al.* (2011) Barriers in the pelagic: population structuring of *Calanus helgolandicus* and *C. euxinus* in European waters. *Mar. Ecol. Prog. Ser.*, **428**, 135–149.