

FISH & CHIPS



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Content

1	Project execution.....	4
1.1	Project objectives.....	4
1.2	Participants of the consortium.....	5
1.3	Description of the work performed and end results	6
	References	15
1.4	Description of methodologies and approaches employed	16
1.5	Achievements of the project related to the state-of-the-art	17
1.5.1	Advances against the state-of-the-art	17
1.5.2	Impact on the industry sector.....	17
1.5.3	Impact on the research sector	17

1 Project execution

1.1 Project objectives

Sustainable development is a fundamental goal of the European Union and loss of biodiversity is emphasised as one of the main threats to it. However, biodiversity and ecosystems of European Seas are under human impact, such as pollution, eutrophication, and overfishing. Therefore it is necessary to monitor changes in biodiversity and ecosystem functioning.

Aim of the “Fish & Chips” project is the development of DNA chips for the identification of marine organisms in European Seas as a cost effective, reliable and efficient technology in marine biodiversity and ecosystem research. Many marine organisms, such as (1) fishes and invertebrates, including their eggs and larvae, (2) zoo- and phytoplankton, and (3) benthic invertebrates, are often difficult to identify by morphological characters.

Since classical microscopy methods are extremely time consuming and require a high degree of taxonomic expertise, this basic step of identification is often the major bottleneck in marine biodiversity and ecosystem research.

Currently, DNA chips are mostly used as research tools for gene expression analysis [1], [2]. Their use for the identification of organisms is not yet widely distributed. Only a few studies on mammals [3], bacteria [4], and viruses [5] have been reported. The “Fish & Chips” project aims to demonstrate that DNA chips can be a reliable tool for the identification of marine animals and phytoplankton. These chips will facilitate research on dispersal of ichthyoplankton, monitoring of phytoplankton, and identification of bioindicators as well as prey in gut contents analysis.

The “Fish Chip” comprises capture oligonucleotides for fishes of the North Sea, Baltic Sea, North-Eastern Atlantic, Mediterranean and Black Sea. Its first version, a chip which allow the identification of 46 fish species, will be evaluated in terms of facilitating ichthyoplankton community studies, as well as investigations of dispersal of fish eggs and larvae.

The “Phytoplankton Chip” focuses on unicellular algae from the North Sea. This DNA chip will enable monitoring of biodiversity, especially for pico- and nanoplankton species which lack morphological features for identification. The detection of harmful algae blooms is also a potential application.

The “Invertebrate Chip” focuses on important prey species of demersal fishes in the Mediterranean and North Sea, as well as on bioindicator organisms, such as polychaetes, which are difficult to identify by morphological characters.

To achieve this goal a combined biological and technical approach has been initiated: The biological material will be sampled by marine biologists. The next step is the sequencing of suitable molecular markers for probe design. The technical part consists mainly in constructing gene probe libraries and determining their specificity. This will be done by biotech research centres in connection with SMEs engaged in DNA chip technology. Therefore the project has the potential to bring Europe’s marine biotechnology to the forefront of this field.

1.2 Participants of the consortium

The project consortium has been established by several partner meetings to achieve a reasonable and complementary consortium structure. The amount of eleven partners in relation to the total costs of the project shows that the main intension of the project is to strongly use the financial resources in a highly effective and adequate way. The following table shows their main contribution of each partner to the project.

Participant name	Main contributions
Centre for Applied Gene Sensor Technology (CAG), University of Bremen	Sampling of fishes in northern European Seas, tissue storage, DNA extraction, amplification of molecular markers and sequencing (fish and invertebrates), bioinformatics and hybridisation experiments
PicoRapid Technologie GmbH	Chip production (task taken over by CAG after May 2004)
Universidad de Oviedo	Sampling of fishes in the western Mediterranean and Gulf of Biscay, tissue storage, DNA extraction, amplification of molecular markers (fish); participation in hybridisation experiments
InfoConsult Gesellschaft für Informationstechnik mbH	Project Management
Prokaria	DNA extraction, amplification and sequencing of molecular markers
Hellenic Centre for Marine Research	Sampling of fishes in the eastern Mediterranean, tissue storage, DNA extraction, amplification of molecular markers (fish); participation in hybridisation experiments
Stiftung Alfred Wegener Institut für Polar und Meeresforschung	Sampling in the North Sea, tissue storage, DNA extraction, amplification of molecular markers (phytoplankton) and hybridisation experiments
Centre National de la Recherche Scientifique - Délégation Languedoc-Roussillon	Sampling of fishes in the western Mediterranean, tissue storage, DNA extraction, amplification of molecular markers (fish) and hybridisation experiments
Exiqon A/S	Development of universal LNA chips, production of LNA-enhanced oligonucleotides and hybridisation experiments with universal LNA chips
Alma Mater Studiorum – University of Bologna	Sampling of invertebrates and fishes in the Adriatic Sea, tissue storage, DNA extraction and amplification of molecular markers (zooplankton) and hybridisation experiments
Mustafa Kemal University, College of Fisheries and Aquaculture	Sampling of fishes and invertebrates in the eastern Mediterranean and Black Sea, tissue storage, DNA extraction and amplification of molecular markers (fishes)

Special attention was drawn to the involvement of SMEs. The *Fish&Chips* project will strengthen in this way the competitiveness of the involved European companies and will foster their leading roles on the European market.

For more detail about the *Fish&Chips* project and the consortium please see: <http://www.fish-and-chips.uni-bremen.de>.

1.3 Description of the work performed and end results

Biological material was sampled in European Seas (Fig. 1-1) and mitochondrial as well as nuclear genes were sequenced as molecular markers to enable probe design for the microarrays. Partial sequences of mitochondrial genes are used as markers for fishes (16S rDNA, COI, *cyt b*) and invertebrates (16S rDNA, COI), whereas the nuclear 18S and 28S rDNA genes are used for phytoplankton.

To share the data between the project partners, an on-line data base has been implemented containing all sequences and relevant information of the samples.

Target DNA has been end-labelled by PCR with the fluorescent dye Cy5 and hybridisation of target DNA is detected by the help of an Axon fluorescence scanner.

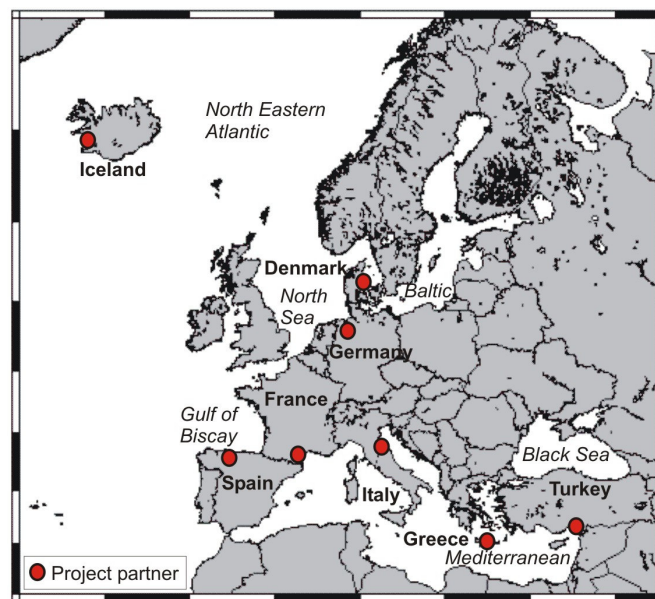


Fig 1-1 Location of the project partners (= sampling areas).

"Fish Chip"

16S Fish Chip

Two version of a "Fish Chip" based on 16S sequences have been developed. A preliminary "16S Fish Chip" contained oligonucleotide probes for 11 commercially important fish species that have been designed based on 230 sequences obtained from 27 species. They have been tested for specificity *in silico* against 1211 background sequences of more than 380 fish species.

Positions of the oligonucleotide probes in the 16S rDNA fragment used for probe design are given in figure 1-2. Binding sites of probes for *Engraulis encrasicolus*, *Sparus aurata*, and *Trigla lyra* are located in the variable region *j*, whereas hybridisation of probes for *Boops boops*, *Helicolenus dactylopterus*, *Lophius budegassa*, *Pagellus acarne*, *Scomber scombrus*, *Scophthalmus rhombus*, *Serranus cabrilla*, and *Trachurus trachurus* occur in the variable region *l*.

All single target hybridisations of the Cy5-labelled 16S rDNA fragment gave true-positive fluorescence signals for the corresponding probe. Only three probes gave eight very weak false-positive signals for single target hybridisations, but the values of these false-positive signals were much lower than the true positive-signals. Single non-target hybridisations showed 48 very weak false-positive signals, representing 25% of 154 possible cross-hybridisations. These false-positive signals were at least one order of magnitude weaker than the true-positive signal. Considering all possible cross-hybridisations, only 18% showed

usually very weak false positive signals (Fig 1-3).

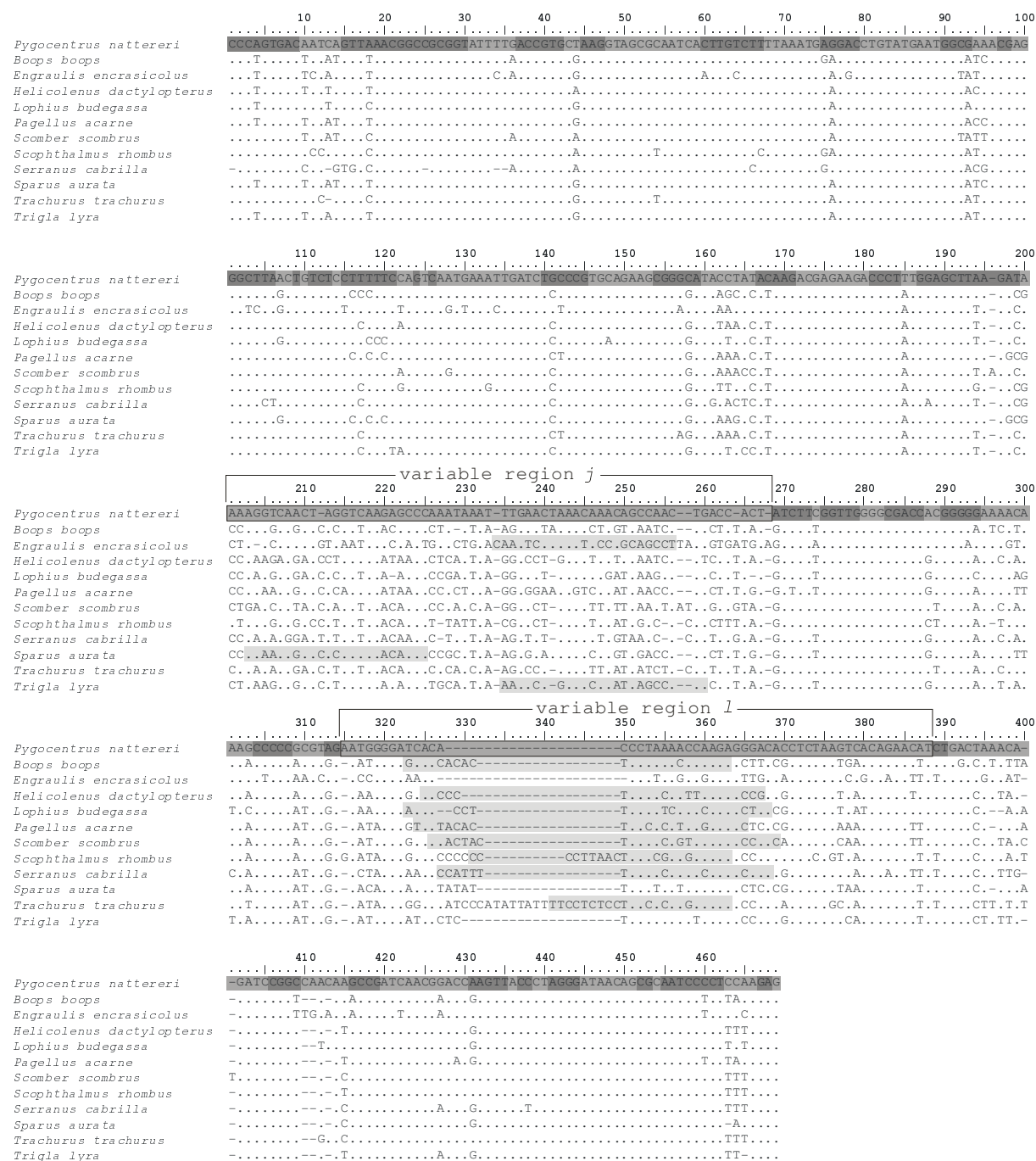


Fig 1-2 Alignment (5'>3') of representative 16S rDNA sequences from the target species with binding sites (light grey) of probes (5'>3'; probes hybridise to the reverse complementary target strand). Double-stranded (dark grey) and single stranded regions (grey) of the secondary structure are indicated in the reference sequence of *Pygoplites nattereri* (Accession number: U33590).

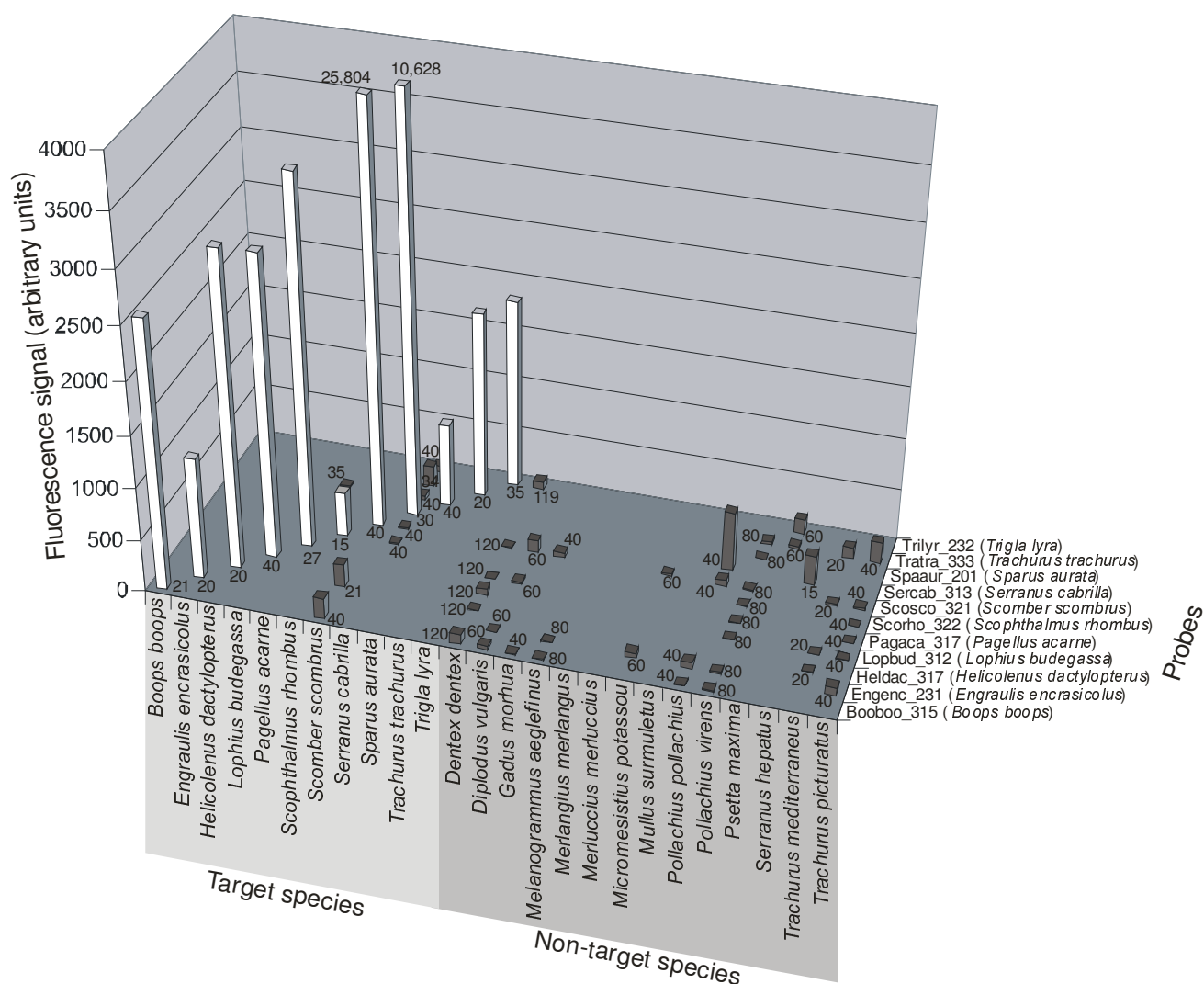


Fig 1-3 Signals of single 16S target and non-target hybridisations. White bars represent true-positive signals; false-positive signals are shown as grey bars. Numbers at the basis of the bars indicate the number of measured spots.

The second version of the “16S Fish Chip” includes 75 probes of 16S rDNA for the identification of 46 marine fish species. Design of the 16S rDNA probes is based on an alignment of 406 sequences of 50 species. Additional probes have been designed on the basis of 470 COI sequences from 48 species and 366 cyt *b* sequences from 43 species.

The targets tested so far show very specific binding to the corresponding 16S probe (Fig. 1-4). Only a few 16S probes show weak false-positive signals or are only specific for a certain group of fishes (e.g. family level).

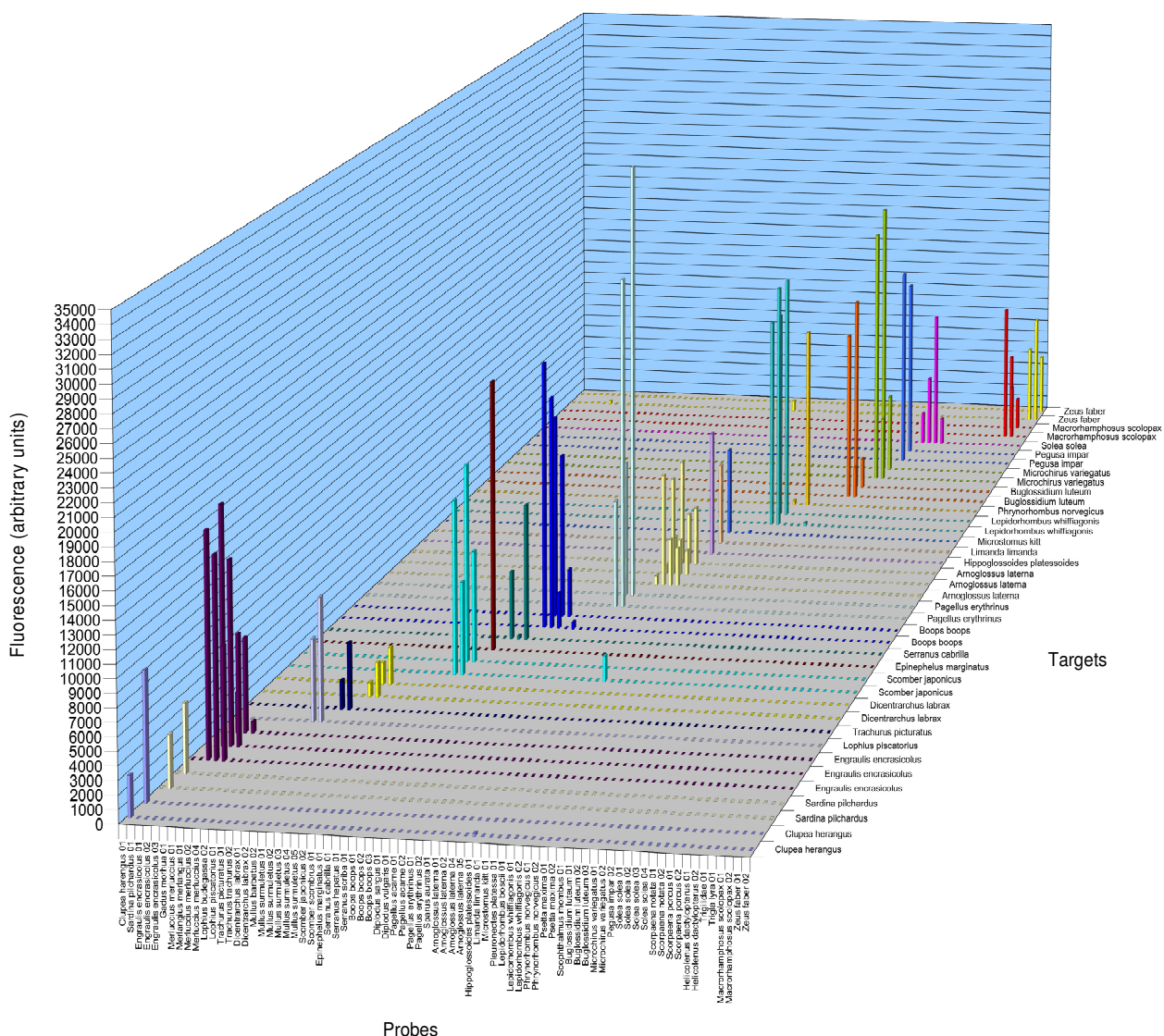


Fig 1-4 Specificity test of the “Fish Chip” by single target hybridisations with Cy5-labelled fragments of the 16S rDNA.

COI Fish Chip

Probes designed on the basis of COI sequences have been tested in hybridisation experiments. From 70 individuals of 17 fish species (13 flatfish, Order Pleuronectiformes, and 4 codfish, Family Gadidae), caught in the North Sea, the COI genes have been sequenced. By multiple alignments of all sequences obtained regions were selected for designing a total of 34 capture oligonucleotide probes which were experimentally evaluated which regard to specificity and sensitivity. Interestingly, the number of mismatches does not relate to the specificity of the hybridisation signal. Furthermore, probes of equal length, GC-content and melting temperature exhibit different hybridisation efficiencies, as the example below indicates. Here, the hybridisation signals of six different capture probes, three each targeting the same species, are shown in hybridisation experiments to these two species (Fig 1-5).

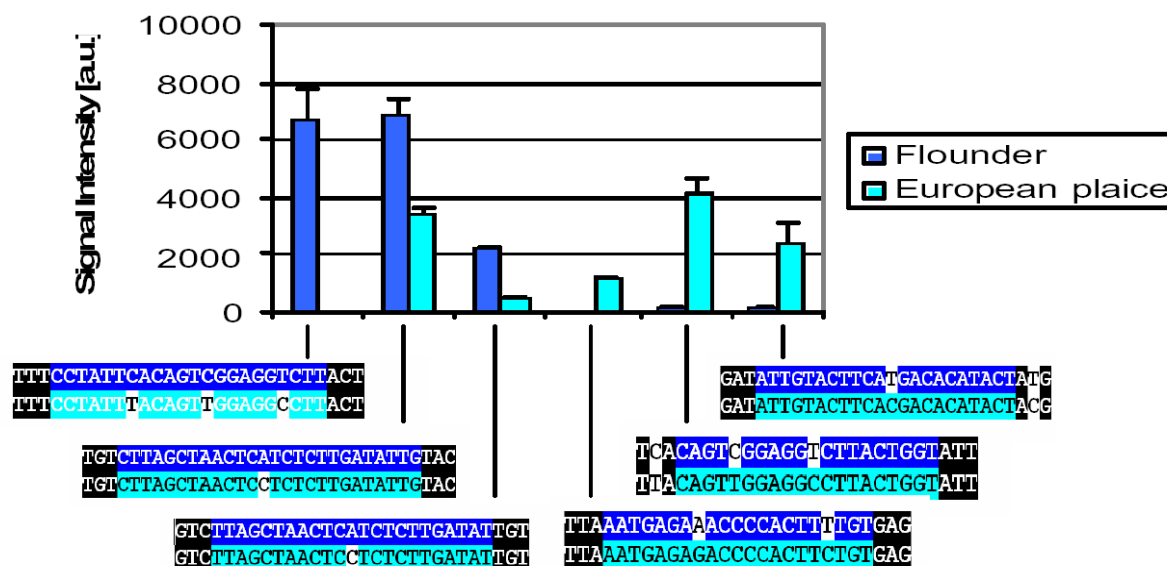


Fig 1-5 Signal intensity of capture probes with different numbers of mismatches

From the 34 capture probes designed to discriminate between 8 fish species, a set of 24 has been selected according to their specificity and hybridisation efficiency (Fig 1-6). The target DNA was PCR amplified using one Cy5 -labelled primer resulting in a 542 bp long fragment. Cross-hybridisation was measured with 10 nM PCR products each from all of the 8 species, one at a time.

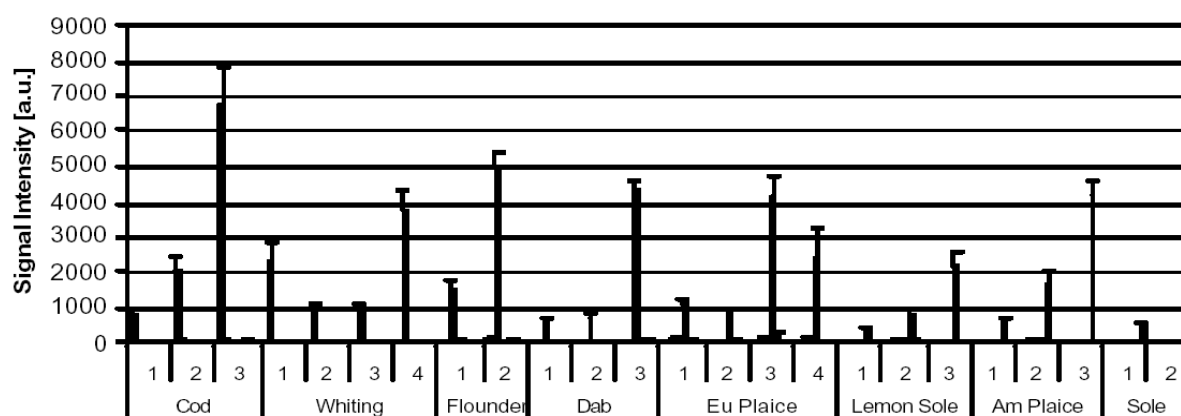


Fig 1-6 Specific hybridisation signals of 10 nM PCR-products of each fish species

A 10 nM solution of the PCR-product generated from flounder (*Platichthys flesus*) was hybridised to 5 Flounder-specific probes, as well as probes representing all the other fish, positive and negative control probes. The example shown in Fig 1-7 (an excerpt from a DNA microarray) demonstrates the signal difference between true-positive and false-positive spots. In this case only the two best capture probes were selected for further routine analyses.

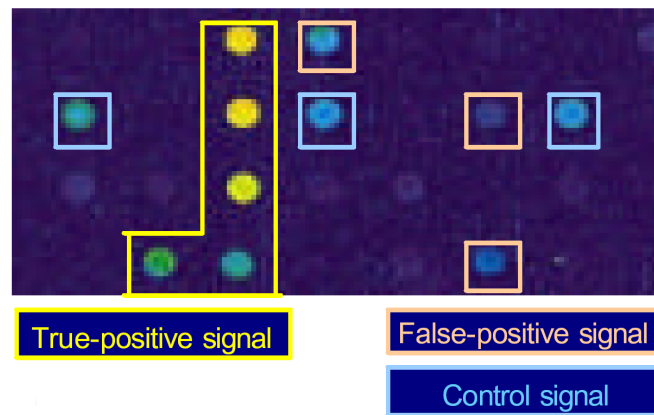


Fig 1-7 Example of DNA microarray hybridisation

One of the reasons for the astonishingly strong differences in the hybridisation efficiency of the capture probes seems to be the distance between the binding site of the probe and the labelled 5'-end of the target DNA. Fig. 1-8 demonstrates this observation: the shorter this distance, the higher the signal intensity.

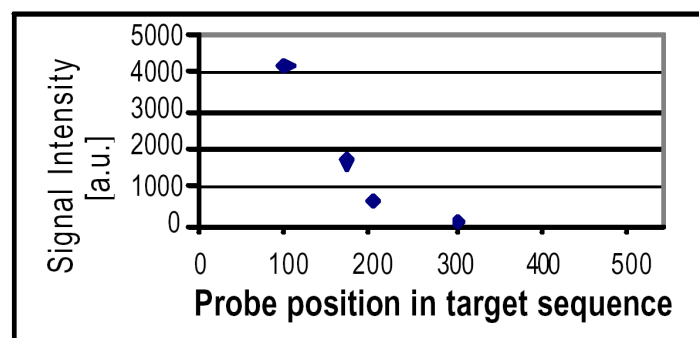


Fig 1-8 Signal intensities of probes in comparison to their location on the target DNA

By designing several capture probes for each fish and by strictly optimising them in respect of their biological specificity and by technically realising semi - quantifiable hybridisation signals a microarray for the simultaneous identification of fish species has been constructed. Because of the inner-species variability and the fact that the more individuals per species are sequenced the more haplotypes will be observed, and because cross-hybridisation effects can hardly be eliminated at least in DNA samples of high complexity, defining capture probes for more and more species to be analysed per one chip is obviously limited. These limits of detection and discrimination abilities of the “Fish Chip” are presently under investigation. These further experiments will have to reveal the practical value of the microarray approach in respect of how many species can be identified in parallel by the help of one single chip, whether or not complete plankton catches can be analysed, to what extent the different species in one sample can be quantified, and what costs are to be expected if using this technology for routine applications.

“Invertebrate Chip”

Based on 149 sequences (495 bp) of a fragment of the mitochondrial COI gene from five invertebrate species, capture oligonucleotides have been designed. These species are also involved in the species list for 16S sequences. The invertebrate chip was tested with 30 16S samples from 15 target species and ten COI samples from five target species, showing very good results (Fig 1-9). The targets tested so far show very specific binding to the correspond-

ing probe. Only very few probes are showing weak false positive signals. Probes for two species showed neither specific nor unspecific binding.

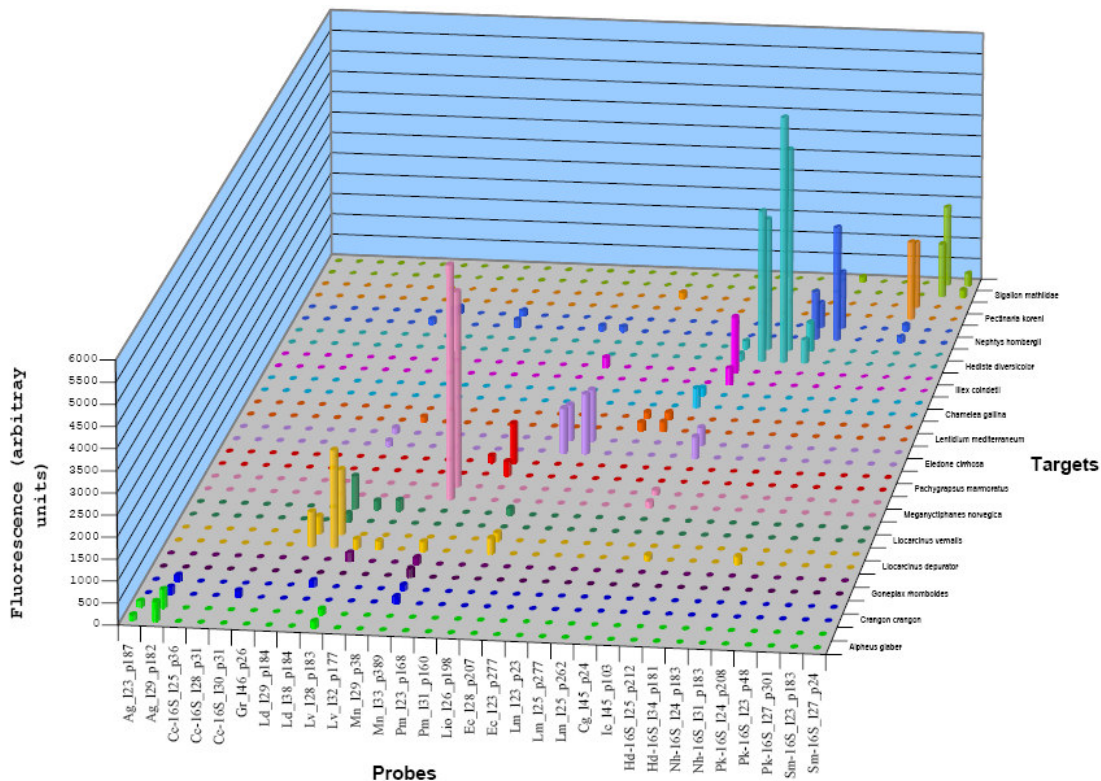


Fig 1-9 Specificity test of the “Invertebrate Chip” by single target hybridisations with Cy5-labelled fragments of the 16S rRNA gene and the COI gene

“Phytoplankton Chip”

Harmful Algae Blooms (HABs) are threatening humans, ecosystems, fishery, tourism and aquaculture and their occurrence in mixed phytoplankton assemblages is often difficult to detect. The genus *Alexandrium* has undergone steady taxonomic revision and identification of its species has been confused because of overlapping morphological features and minute differences. The design of molecular probes from the 28S rDNA has shown great potential for distinguishing of species or even clades, but whole-cell hybridization methods are tedious and time-consuming. In this part of our experiments, probes were evaluated for differentiation of several species belonging to the genus *Alexandrium*. Four probes for the *A. tamarense* “species complex” were tested on the microarray. The specificity of the probes was tested by hybridization with 28S PCR-fragments of pure cultures and by analysis from spiked field samples from the Weser estuary (German Bight).

Figure 1-10 shows the 28S probes for the *A. tamarense* “species complex”, hybridised with specific PCR-fragments. All probes showed overall good and specific hybridisation results. No probe gave unspecific cross-reactions, neither with the closely related *A. ostenfeldii* PCR-fragment nor with PCR-fragments from the other clades that were examined in this study.

The probes ATNA01 and ATNA02 performed specific with high signal-to-noise-ratios for all three target strains from the North American Clade. It turned out that the shifted probe ATNA02 showed slightly better ratios, nevertheless, both performed very satisfactory. Hybridisation with three strains of the Western European Clade resulted in excellent signals for the ATWE03 probe, showing great discriminative potential and sensitivity with signal-to-noise-ratios ranging from 124-252. The same can be reported for the signals for probe ATME04 evaluated with four strains of the Mediterranean Clade, which performed with values from 80-190.

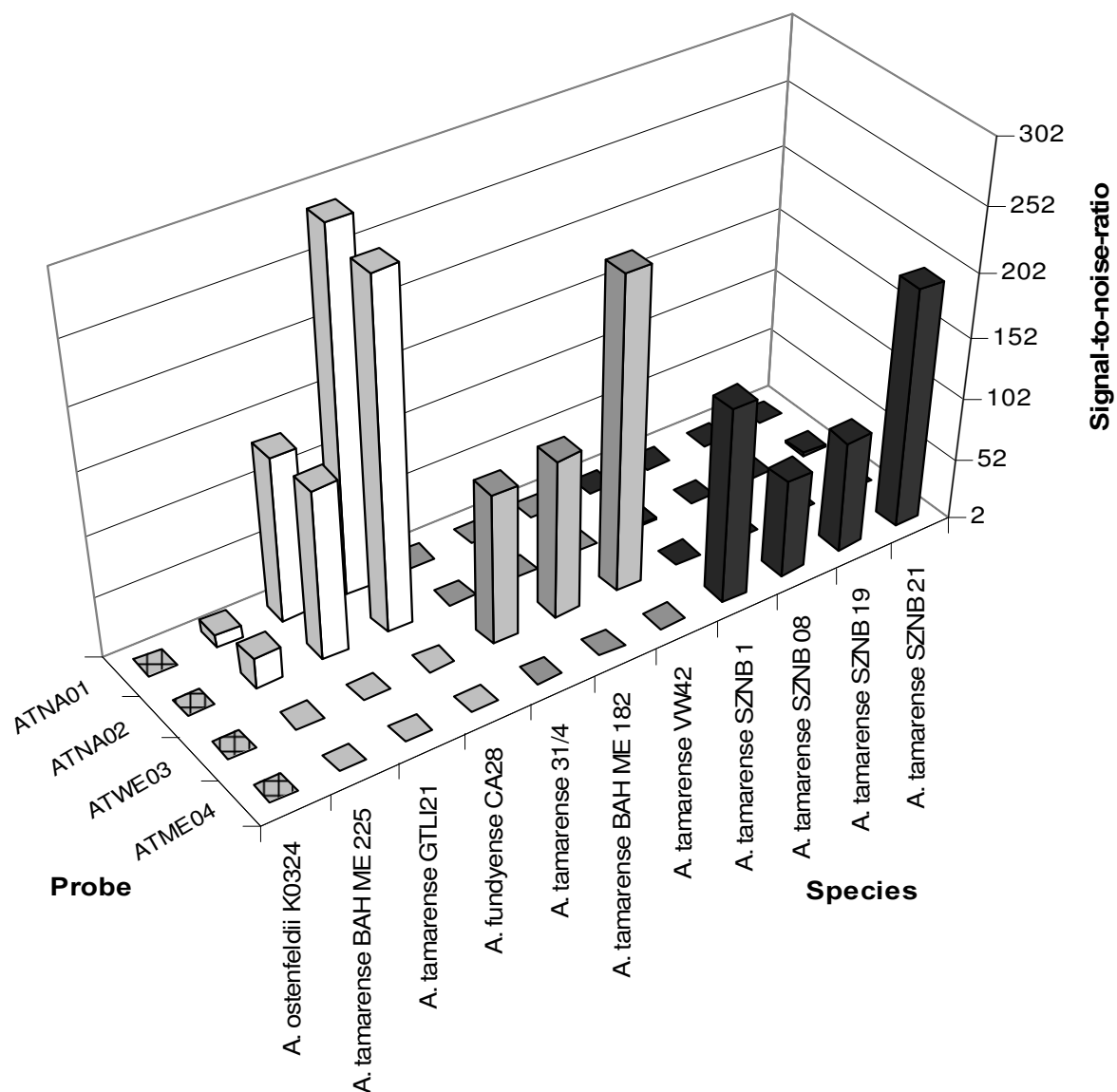


Fig 1-10 Specificity tests of the 28S probes for the *A. tamarensis* "species complex"

An 18S rRNA gene-targeted oligonucleotide microarray consisting of 21 probes for the characterisation for microbial picoeukaryotic communities in marine environments was developed for detection of members of the Prasinophyta. Probes from other hybridisation methods were adapted and new probes were designed for novel groups where no probes were available based on 18S sequences. Evaluation of the probe set was done with 18S PCR-fragments from 20 unialgal reference cultures. The results demonstrated the suitability of the microarray as a reliable tool for fast and efficient monitoring.

Probe specificity was tested under the same conditions (same hybridisation temperature and washing conditions for all probes and chips). In general, hybridization results of this chip showed high specificity and sensitivity (Fig. 1-11). Signal-to-noise-ratios of perfectly matched targets hybridized to their specific probes showed different values, demonstrating that the intensities of individual probes vary strongly in their sensitivity.

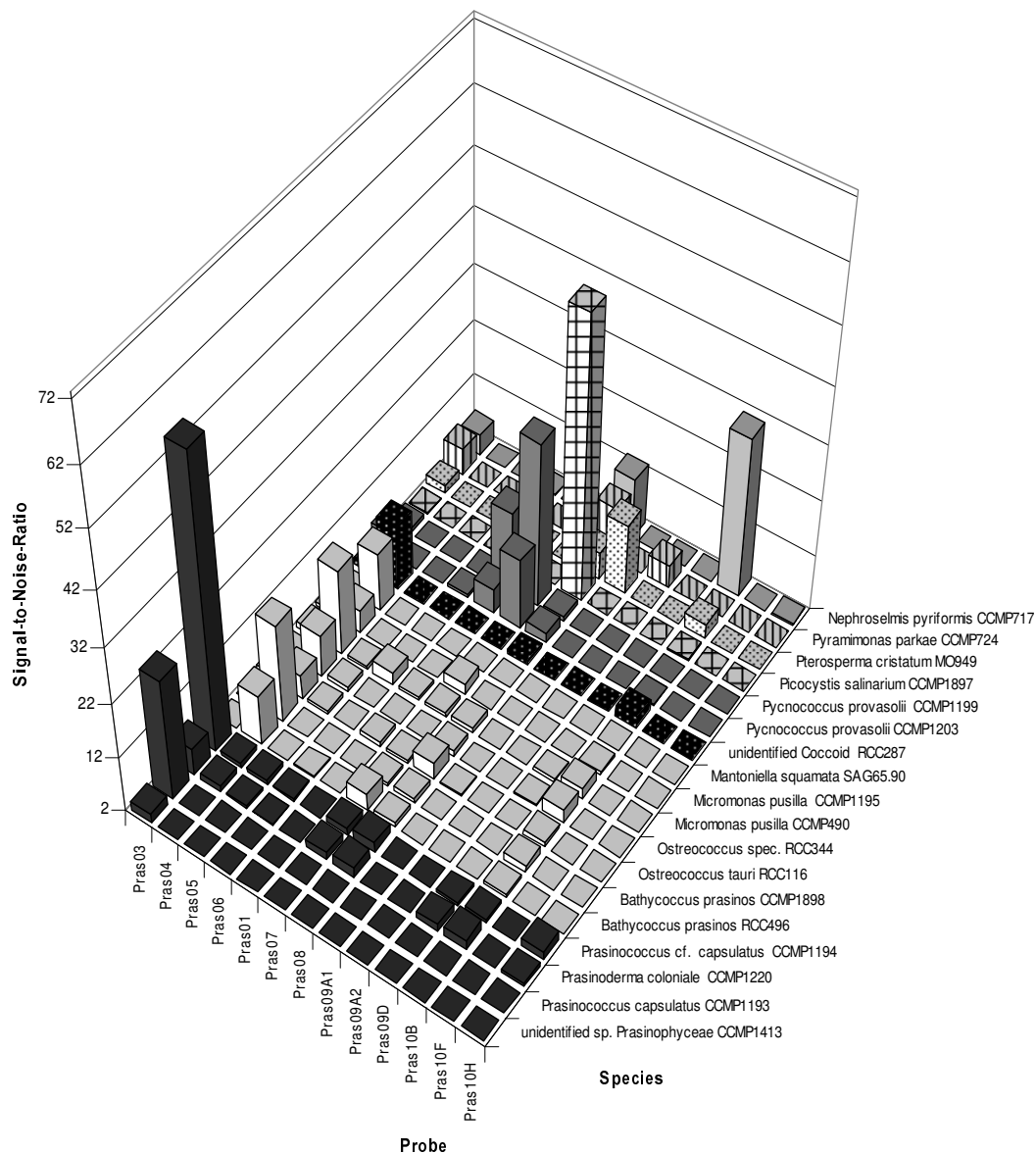


Fig 1-11 Hybridization results of 21 reference cultures to six Prasinophyta probes.

Conclusions

The results we obtained for the three DNA microarrays under development show that this approach is seemingly feasible and that the genetic identification of marine organisms by this method is a realistic approach. The reliability under routine application is still under investigation.

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1.4 Description of methodologies and approaches employed

DNA microarrays have the potential to identify hundreds of species at once. These bio-chips made from glass or other materials, contain up to many thousands of spots in which oligonucleotides are fixed to the chip surface. They serve as capture probes to bind by hybridisation complementary target sequences from the biological samples. These sequences have previously been selected to be specific for the different species. The DNA is being extracted from the organisms, PCR-amplified, labelled and hybridised to the capture oligonucleotides on the chip. Alternatively rRNA can be extracted and the signals can be interpreted as semi-quantitative. Scanners are used to measuring the fluorescence of every spot (Fig. 1-12).

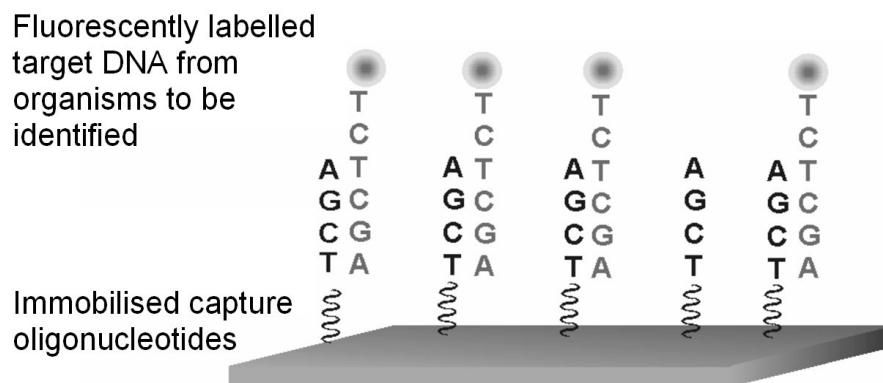


Fig 1-2 Schematic illustration of a DNA microarray.

1.5 Achievements of the project related to the state-of-the-art

1.5.1 Advances against the state-of-the-art

In course of the “Fish & Chips” project, DNA microarrays for the identification of European marine fishes, benthic invertebrates, and phytoplankton have been developed. To our knowledge, this is the first application of DNA microarray technology for the identification of fishes and benthic invertebrates. The development of DNA microarrays for the identification of phytoplankton has been initiated already in earlier projects by the project partner AWI. However, so far no publication about the development of DNA microarrays for the identification of European marine fishes, benthic invertebrates, and phytoplankton is recorded in scientific literature. This field of research is still in its infancy and therefore the results obtained in the “Fish & Chips” project are advances against the state-of-the-art.

1.5.2 Impact on the industry sector

The results of the “Fish & Chips” project have shown that an identification of more than 40 fish species with a DNA microarray is feasible. Therefore, microarrays could be a useful tool for the identification of fish species in the seafood industry and fisheries control.

The work on the “Phytoplankton Chip” has shown that it can detect toxic algae. Therefore, such a microarray can be used to detect the early development of harmful algae blooms (HAB). This is important for aquaculture as well as fisheries (e.g. mussels).

1.5.3 Impact on the research sector

The obtained results have shown that the identification of early life stages of marine fishes and invertebrates, as well as unicellular algae by DNA microarrays is feasible. This might encourage more research groups to use and develop DNA microarrays for the identification of marine organisms in ecological research.