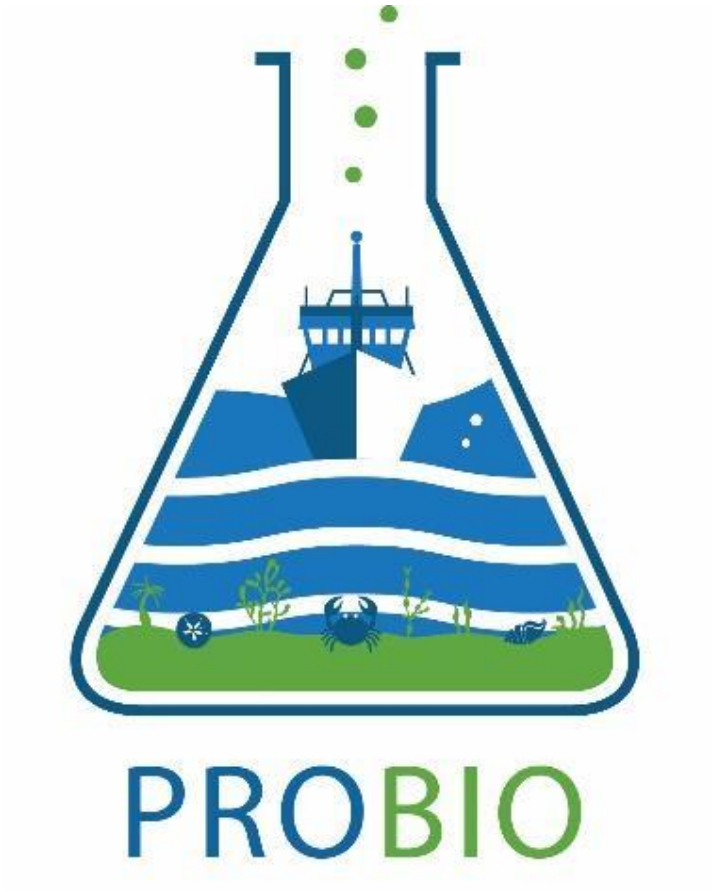

PROspection for BIOactive compounds in the North Sea

Deliverable 5.1 Final PROBIO report
Date: October 2022

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BLAUWE CLUSTER
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VLIZ

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KU LEUVEN

UNIVERSITEIT GENT

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1. Summary

Marine environments offer a wide variety of bioresources containing potential bioactive compounds. The potential of bioactive compounds from these marine bioresources is currently hugely underexplored in our seas and oceans, and will offer a 'sea of opportunities' in several sectors involved with the research, development and production of functional additives and products. Primary and secondary metabolites in marine organisms are of particular interest because they have unique properties and a broad valorisation potential in e.g. pharma, cosmetics, agriculture, nutraceuticals, chemicals and functional bio-based materials. The **PROspection for BIOactive compounds in the North Sea (PROBIO) project** is focusing on the discovery and characterisation of new bioactive compounds derived from local North Sea species.

The **identification and selection of 50 North Sea species** with a commercial potential for bioactive compounds was the starting point for further sampling and screening activities in the PROBIO-project. The compilation of the species list was based on an assessment of the relevant scientific literature, with regard to marine taxonomic groups and compounds with potential biological activity, resulting in 14 taxonomic groups. Three selection criteria were used to shorten this exhaustive list to a smaller list of 147 species, which were abundance, species size and possibility for easy determination. The Strategic Advisory Board (SAB) was involved in the formulation of criteria and the species selection procedure for the species shortlist. Criteria that were considered were the potential of cultivation and the available biomass in sufficient replicate sites, resulting in a final shortlist of 50 species. For each of these species, a 'bioprospection index card' was established providing information on potential compounds, cultivation opportunities and spatial distribution. The **PROBIO sampling campaigns** carried out by VLIZ took place in four seasons between May 2020 and April 2021 and resulted in the collection of all 50 preselected species through various sampling techniques, i.e. van Veen grab, beam trawl, horizontal plankton net (WP3) and manual collection with the help of the VLIZ scientific diving team. Species were collected at 40 different locations (nearshore and offshore) on the Belgian part of the North Sea.

A very concise and high-throughput protocol for **fractionation of PROBIO samples** was established and performed on 40 samples with high biodiversity and chemical diversity, covering a total of 31 species, and resulting in a total of 9888 fractions from marine organisms of the Belgian part of the North Sea. A workflow was established between VIB responsible for fractionation and KU Leuven and UGent for the bioassay screening. From these 31 species, 11 positive hit fractions were subfractionated for further bioactivity testing. The **analytical screening** resulted in a highly valuable dataset of 374 samples of 50 different species from the Belgian part of the North Sea, covering a total of 10 phyla. For each sample three profiles (UPLC-HRMS negative and positive ionization mode and GC-HRMS) were produced resulting in a total of 1122 analyses/chromatograms. In total more than 20K compound ions (for LC-MS) and +9K features (for GC-MS) were measured for this set of samples. For each compound ion a set of information is available: accurate mass, ion mobility data (for LC-MS only), fragmentation pattern, and abundance/occurrence through species, phylum, geographical location and season. Raw data was converted to an open source format (mzML) and can be requested by email. A link will then be provided giving access to download the data. The processed data for both LC-MS and GC-MS is available at the PROBIO project page.

Electrophysiological assays were carried out for 19 samples of 13 species. From a set of targets (ion channels or receptors) that are associated with certain physiological processes in humans and animals, 3 targets were selected for this project: adult muscle type nicotinic acetylcholine receptor (nAChR), voltage-gated muscle type sodium channel (Nav1.4) and voltage-gated potassium channel (Kv1.3), after advice from the Strategic Advisory Board thus aiming at a broad valorisation. The active effect of the

metabolites on these targets is measured as potential difference in oocytes of the model organism *Xenopus laevis*. Partial or complete blockage of the nAChR channel and the Kv1.3 channel was detected for several fractions of several samples. The assays on the Nav1.4 channel were carried out for a limited number of samples, also reflecting the limited number of fractions and samples for which a partial blockage of the Nav1.4 channel was detected. Based on the results, two positive fractions from *Asterias rubens* (F81) and *Ophiura ophiura* (F65) were subfractionated for further bio-activity testing in WP3. However, due to limited resources, assays on subfractions have not been carried out in WP3. The results of the fractions from *O. ophiura* are similar to the results of *A. rubens*, and since these two organisms are closely related on the phylogenetic tree it is plausible that they have common bioactive molecules (e.g. saponins). Saponins are molecular structures that are known to have anti-bacterial and anti-viral properties. These species use saponins as a defense mechanism against predators and diseases, which make these compounds a very interesting subject to study for valorisation.

Antimicrobial activity against *Candida albicans*, *Staphylococcus aureus*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* (for some cases) was carried out for 31 species. Antimicrobial tests included the determination of minimal inhibitory concentration and minimal bactericidal/fungicidal concentrations. In the second research phase, the antibiofilm activity of selected compounds was evaluated. Antimicrobial activity is found in a wide range of organisms and fractions but is predominantly found in early (F2-F4) and late (F80-F85) fractions. This pattern occurred in a wide taxonomic distribution suggesting that antimicrobial activity in these 'early' and 'late' fractions is aspecific, and possibly attributable to high salt concentration ('early' fractions) and the presence of surfactants ('late' fractions). Follow-up research should be carried out to confirm this. Based on the results, 8 positive hits from 5 species (*Alcyonium digitatum*, *Echiichtys vipera*, *Ophiura albida*, *Ophiura ophiura, Liocarcinus depurator*) were identified for further subfractionation. Several subfractions revealed a positive hit again, showing that subfractionation of positive fractions is possible without losing the bioactive compound, and resulting in a much less complex subfractions containing only a few compounds of which one of them is the bioactive compound. Some results also show that the antimicrobial activity of positive fractions was lost during subfractionation. The reason for this is at present unclear, but could be related to unstable compounds (degradation) or too low concentrations after subfractionation. Due to limited resources, not all of the promising fractions could be subfractionated and further testing for growth inhibitory and killing activity towards planktonic cells should be continued in follow-up research, especially the hit fractions outside the 'early' and 'late' regions (*Asterias rubens*, *Echinocardium cordatum*, *Ophiura albida, Ophiura ophiura, Sargassum muticum, Lanice conchilega, Mytilus edulis*).

The hit species found from the bioactivity screening were cross-linked with the bioprospection index cards in order to identify the **most likely application(s)** for the detected active fractions. For some species, evidence of bioactivity was already reported in literature, while for others the discovery is quite novel. Moreover, whenever cultivation opportunities were known for a certain species, the corresponding publication was included in the bioprospection index cards. For five species (*Asterias rubens*, *Echinocardium cordatum, Ophiura ophiura, Sargassum muticum* and *Ophiura albida*) and five applications (cosmetics, pharmaceuticals, antifouling, functional ingredient and hygienic packaging), a use case was developed. The species were chosen based on the most promising results from the antimicrobial and electrophysiological assays. This resulted in **a set of five use cases for promising hit species** that were described in a fact sheet.

From an application perspective, the Strategic Advisory Board identified the use case "antifouling solutions in offshore and onshore aquaculture facilities" as most promising. In a brainstorm session the

assumptions that need to be validated regarding problem fit, solution fit and business model fit were identified. This resulted in a concept note for a feasibility study. However, it became clear that there is still a large gap between the research results and the industrial applications. **Therefore, a next step needs to be taken by the research partners in identifying and purifying the compounds that were found in the "hit" fractions and sub-fractions. Fractionation of the active mixtures to generate subfractions with a less complex metabolic composition is highly desired in order to perform a more targeted study on the biotechnological potential of the compounds produced by a certain organism. This knowledge is important for the industry in order to be able to register new products and to develop new processing technologies/partnerships.**

2. The PROBIO project

The Flemish sector of blue biotechnology is immature and in need of fundamental research to make smart choices, mainly in the context of the selection of species/bioresources in the Belgian part of the North Sea and economic strengths of Flemish companies. Marine environments offer a wide variety of bioresources containing potential bioactive compounds. The potential of bioactive compounds from these marine bioresources was hugely underexplored in our seas and oceans, and offers a 'sea of opportunities' in several sectors involved with the research, development and production of functional additives and products. Primary and secondary metabolites in marine organisms are of particular interest because they have unique properties and a broad valorisation potential in e.g. pharma, cosmetics, agriculture, nutraceuticals, chemicals and functional bio-based materials.

Nevertheless, the biodiscovery of new bioactive compounds has so far been limited in the North Sea region. Through a value chain approach, the project stimulates the upscaling of aquaculture and the expansion of biorefinery sectors and biotech markets at the same time. This by focusing on the discovery and characterisation of new bioactive compounds derived from local North Sea species. The establishment of a reproducible and scalable high-throughput analytic screening of extracts with multi-target bioactivity screening and toxicity assessments is a unique workflow established within PROBIO (Figure 1), combining the strengths of leading research groups of four different institutes in Flanders:

- Flanders Marine Institute (VLIZ)
- VIB-Metabolomics Core Ghent (VIB - MCG)
- Laboratory of Toxicology and Pharmacology at KU Leuven (KU Leuven – LTP)
- Laboratory of Pharmaceutical Microbiology (LPM) at UGent (UGent – LPM)

PROBIO provides a critical first step, providing a knowledge base, which triggers further innovation to foster new commercial developments in various sectors. As such, the expertise gained holds potential for future marine biotech valorisation and innovation projects.

The specific project objectives, linked to the knowledge gaps described in the 5 research work packages are (Figure 1):

1) The identification and collection of **50 North Sea Species** possibly containing bioactive compounds with a commercial potential and the development of "**Bioprospection Index cards**" summarizing existing information of these species on potential compounds, cultivation opportunities and spatial/seasonal distribution (VLIZ).
2) The **high-throughput screening** of extracted compounds and the establishment of an **open-source comprehensive database**, combining structural, (analytical) mass spectral, biological activity information of the compounds and other metadata dedicated to marine organisms, which will be of great value for both the industrial and research community (VIB – MCG).
3) The **identification of pharmacological targets** for the selected bioactive compounds with a commercial potential by performing high-throughput bioassays (KU Leuven – LTP).
4) The **high-throughput screening of the antimicrobial activity** of extracts recovered from North Sea species, showing biofilm-inhibitory and -eradicating activity (UGent – LMP).
5) The identification of **promising applications** of marine bioactive compounds and the stimulation of new **follow-on innovation projects** for biorefinery, aquaculture and biotech applications in Flanders, by the integration of actors from industry and knowledge institutions (VLIZ).

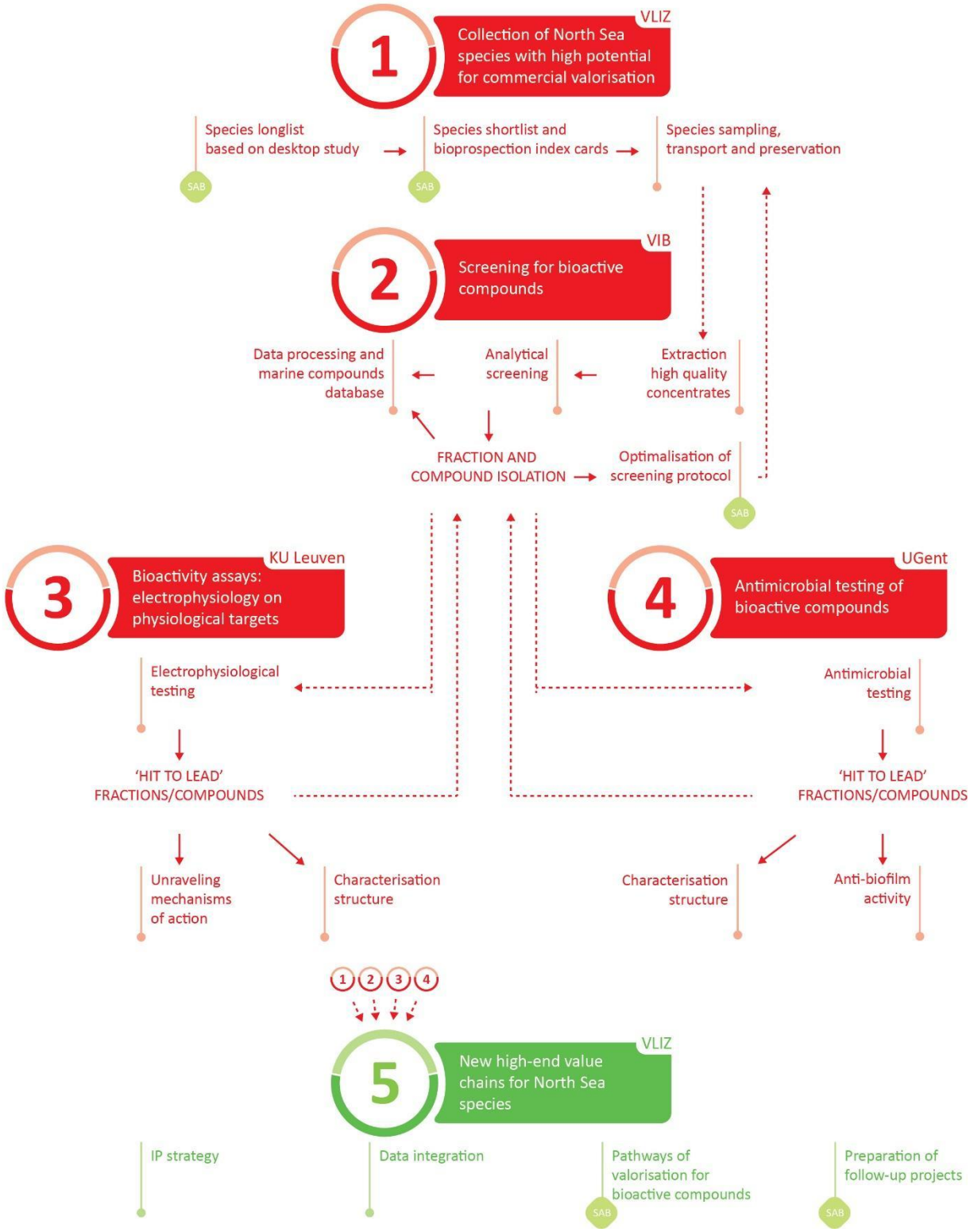


Figure 1: Workflow of the PROBIO project.

3. Methods

3.1. Collection of North Sea species with potential for commercial valorisation

The identification and selection of 50 North Sea species with a commercial potential for bioactive compounds was the starting point for further sampling and screening activities in the PROBIO project. The compilation of the species list was based on an assessment of relevant scientific literature, with regard to marine taxonomic groups and compounds with potential biological activity. The Belgian Register of marine species (BeRMS) was consulted to identify North Sea species of the 14 taxonomic groups identified by the literature search. Three selection criteria were used to shorten this exhaustive list in BeRMS to a smaller list of 147 species (Deliverable 1.1). These criteria are abundance, species size and possibility for easy determination. The Strategic Advisory Board (SAB) was involved in the formulation of criteria and in the species selection procedure for the species shortlist. Based on their input, and on the results of the preliminary sampling that took place in March 2020, a shortlist of 50 species was finalised (Deliverable 1.2). Additional criteria that were considered were the potential of cultivation, the available biomass in sufficient replicate sites. For each of the selected species of the shortlist, a 'bioprospection index card' was established providing information on potential compounds, cultivation opportunities and spatial distribution (Deliverable 1.3). The sources used for compilation of these bioprospection index cards were: EMODnet Biology, LifeWatch, Coastal Portal and the Marine Life Information Network (MarLIN, Tyler-Walters et al. 2022).

The PROBIO sampling campaigns took place in four seasons between May 2020 and April 2021. Throughout the four sampling seasons, all 50 preselected species were sampled during 27 sampling campaigns. Eighteen campaigns were conducted aboard the RV Simon Stevin, two aboard the Ephyra, four on the Spuikom and three at a breakwater in Ostend. Four sampling techniques were employed depending on the location and the targeted species, i.e. van Veen grab, beam trawl, horizontal plankton net (WP3) and manual collection with the help of the VLIZ scientific diving team (in Spuikom, on breakwater, on shipwrecks, on tripods and on moorings). When possible, environmental conditions were measured using CTD in order to trace possible concentration differences of bioactive components. Species were collected at 40 different locations (nearshore and offshore) on the Belgian part of the North Sea (Deliverable 1.4).

3.2. Fractionation

3.2.1. Fractionation of metabolic pools

A fractionation protocol was established and optimized based on a set of preliminary test samples. Based on these tests, a minimum of 100g of fresh material was set as a parameter to include the samples for fractionation. Due to the variety in size and amount of material harvested among the 50 species from the short list, species with enough material were fractionated per season, while other for species with low amount of material the material of different seasons was pooled to reach the necessary amount of material aiming to fractionate as many different species as possible (Deliverable 2.2).

For the metabolite extraction (Figure 2) all fresh material was pooled together and extracted overnight with acetone (2x500mL). The liquid extracts were then pooled together and evaporated to generate the crude extract. This crude material was submitted to solid phase extraction where it was first resuspended in 30mL of MeOH 10% and then loaded on a C18_SPE column preconditioned with 20mL of MeOH and 20mL of MeOH 10%. The C18_SPE column was then eluted with 20mL of MeOH followed by 20mL of DCM. The MeOH subfraction was dried on rotavapor, resuspended in MilliQ water and

filtered through a 0.22µm filter. The volume was then adjusted to 30mL for fractionation on the Autopurification system.

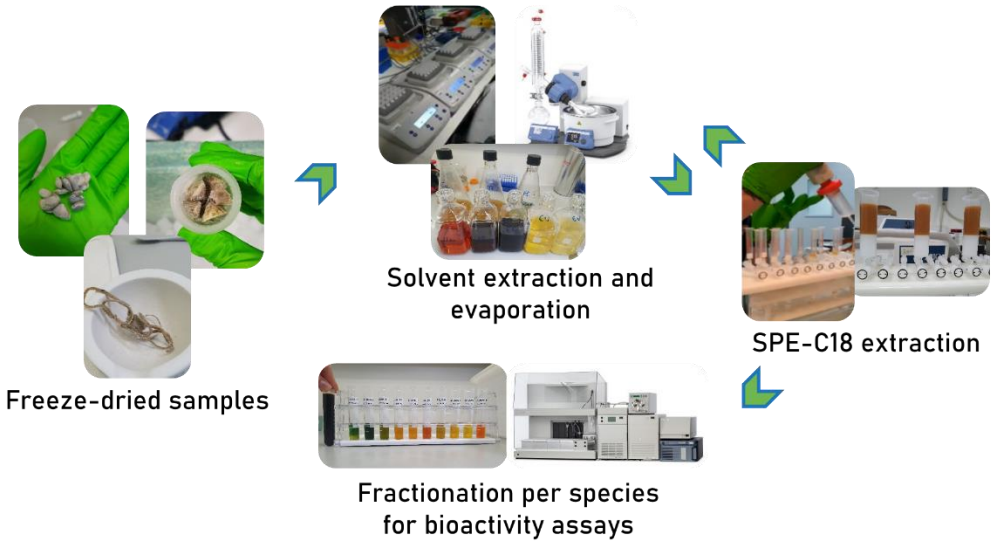


Figure 2: Experimental setup of extractions and fractionations.

For the PROBIO project, a Waters AutoPurificationSystem equipped with a Waters SunFire C18 analytical Column (100Å, 5 µm, 4.6 mm x 150 mm), a SunFire C18 Prep Column, (100Å, 5 µm, 10 mm x 150 mm) and an Acquity QDa Detector was used. A gradient of two buffers was used: buffer A (99/1/0.1 H2O/acetonitrile/formic acid, pH 3), buffer B (99/1/0.1 acetonitrile/H2O/formic acid, pH 3) as follow: 95%A for 0.1 min decreased to 50%A and 50%B in 30 min, then increased to 100%B in 1 min, hold on this buffer mixture for 3min. Subsequent gradient to return to initial conditions and column conditioning time in 6 min, resulting in a chromatographical analysis of 40 min. The flow was 2 mL/min and 10 mL/min for analytical and preparative separation, respectively. Columns were at room temperature for the analysis. QDa detector was set on both positive and negative ionization mode with a mass range from 215 to 1000 Da. The volume of sample injected was 20uL and 9900uL for analytical and preparative runs, respectively. The fraction collection was set as timed event of 23 seconds per fraction with a collector delay of 26 secs, which resulted in 96 subfractions on a single preparative run.

Once a fraction showed a positive hit in the bioactivity assays (WP3 and WP4), it is crucial to track back the metabolic composition of that specific fraction. For that reason, for each fractionation performed and submitted to WP3 and WP4, one batch of similar fractions was stored at MCG-VIB. These fractions were submitted to analytical screening on UPLC-HRMS since they will still contain several tens of compounds (see section 3.3).

3.2.2. Subfractionation of metabolic pools

Each fraction resulting from a preparative fractionation contains several compounds, depending on the complexity of the sample this can be several tens of compounds. When a positive hit is found for one of these preparative fractions in WP3 and 4 (see sections 3.4 and 3.5), it means that one of the compounds in the prep fraction is showing biological activity, but it is impossible to exactly annotate which compound. To determine the real active compound in that fraction, subfractionation is necessary, to separate all compounds present in that lead fraction from each other. This can only be achieved when

higher chromatographic resolution is used (cfr. UPLC Reversed Phase chromatography). The restriction here is that only small volumes (up to 20ul) can be injected, and a limited amount of sample can be loaded. The positive side is that these systems are very reproducible, enabling several repeated rounds of injections (up to hundred) of the sample and resulting in the same high-quality chromatographic resolution. Through the use of a Waters Fraction Manager – Analytical (WFM-A) it is also possible to collect the separated compounds resulting from the high-resolution chromatography, and to collect the same peak in the same vial, enriching each fraction for exactly the same compound and only that compound. This subfractionation will result in fractions that are much cleaner, depending on the complexity of the lead fraction, x number of sub-fractions will be generated for each subfractionation of a lead fraction containing a biological active compound. These subfractions then need to be re-tested in WP3 and 4 to annotate the sub-fraction containing the active compound, and can then be analyzed by HR-LC-MS to determine the accurate mass of the compound(s) present in that sub-fraction (Figure 3).

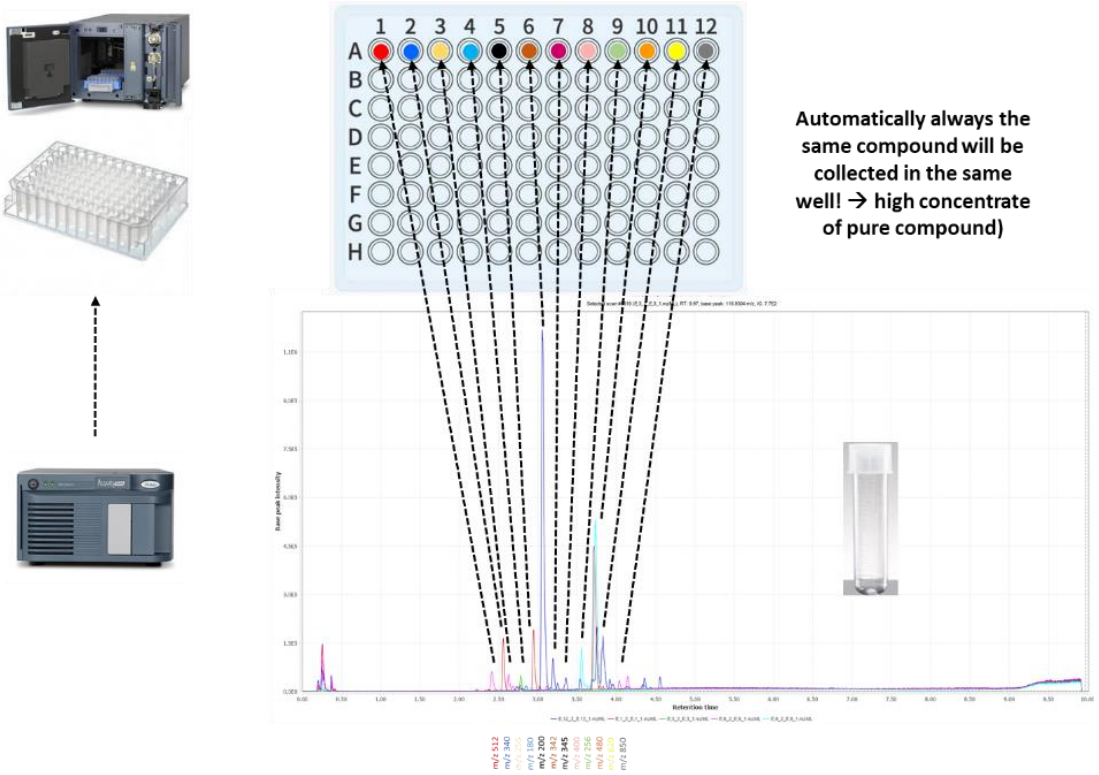


Figure 3: Experimental setup of subfractionation of positive fractions resulting from the initial preparative fractionation. A positive fraction is analyzed through analytical LC-MS and subfractionation is done on the area containing peaks (here between 2.3 and 4.7 minutes).

Based on the elution time and the complexity of each fraction, the elution gradient for subfractionation was adapted. For more hydrophilic compounds (early fractions) the elution gradients were edited in such way the maximum retention was achieved, often resulting in an isocratic elution. For Hydrophobic fractions (late fractions), the gradient was adapted in such way that a high content of organic solvent was used already at the start of the gradient. For each fraction a maximum chromatographic separation of the compounds in that fractions were aimed for. The actual gradients are optimized specifically for that positive fraction and are unique for each subfractionation. For each subfractionation, the whole sample was reinjected in several repeats (depending on the volume left for each prep fraction). All compounds were collected (time-based) in their respective vial, meaning the same compound will always be in the same vial, enriching all compounds after each round if injection. This is possible because

of the high reproducibility of the UPLC system which would not be possible through a conventional preparative fractionation system based on HPLC separations.

An inventory of spare frozen species, non-fractionated grounded material, and spare subfractions is stored at MCG-VIB for future needs on the research about the North Sea biodiversity.

3.3. Analytical screening

The analytical screening is composed with the steps of metabolite extraction, C18 solid phase extraction as a sample clean-up step followed by UPLC-HRMS and GC-HRMS analysis. A total of 1081 samples were processed and pooled to result in 374 samples that were submitted to UPLC-HRMS and GC-HRMS analysis (Deliverable 2.1).

UPLC-HRMS profiling was performed by an LC-MS instrument, which consists of two major parts, a liquid chromatograph (LC) and a mass spectrometer (MS). In the LC, a sample is injected onto a C18 Reversed Phase (RP) analytical column and the different metabolites within the sample are separated based on their affinity for the hydrophobic stationary phase of the column. As mobile phases, water and an organic solvent (acetonitrile) are used (both acidified with formic acid) as a gradient (starting from 100% aqueous to 100% organic). The different molecules within a sample will therefore elute from the column at different retention times. The metabolites that elute from the LC column enter the MS in which the accurate masses of the metabolites are determined. To be able to determine the mass of a metabolite, it needs to be ionized. Here, electrospray ionization (ESI) is used in positive and negative ionization; a soft ionization method at low energy is applied (generally 6eV) which preserves the (protonated or deprotonated) parent ion of a compound (in-source fragmentation does still occur but is minimized). For fragmentation of the molecules, tandem mass spectrometry is applied at higher voltage (generally ramped from 30 to 70eV). This fragmentation is highly reproducible and as such the fragmentation pattern of a molecule can be used for its annotation/identification. After ionization, the masses of all fragment ions will be determined by the MS. In this case, a quadrupole time-of-flight (QTOF) MS was coupled to the LC. With a QTOF, the mass-to-charge ratio of the ions is determined via a time-of-flight measurement. This high-resolution instrument provides accurate mass data, which significantly aids the compound annotation/identification. As output of the LC-MS analysis, a chromatogram showing the total ion current (TIC) recorded by the MS over time is generated for every sample.

GC-HRMS profiling was performed by a GC-MS instrument, which consists of two major parts, a gas chromatograph (GC) and a mass spectrometer (MS). In the GC, a sample is injected into a capillary column and the different metabolites within the sample are separated in the gas phase based on their affinity for the stationary phase of the column. As mobile phase, a chemically inert gas (here, helium) is used. The different molecules within a sample will elute from the column at different times. The time it takes for a certain molecule to travel through the entire length of the capillary column (here, 30 meters) is the retention time of the molecule. As the separation of the sample occurs in the gas phase, the analyzed compounds need to be volatile. To increase the volatility of the analyzed metabolites, they are derivatized (here, trimethylsilylation) prior to injection into the GC. The metabolites that elute from the GC column will enter the MS in which the masses of the metabolites are determined. As output of the GC-MS analysis, a chromatogram showing the total ion current (TIC) recorded by the MS over time is generated for every sample.

MCG-VIB evaluated and established parameters of software packages for high throughput GC and UPLC-HRMS data processing in order to process the dataset of the project.
For UPLC-HRMS data processing of raw chromatograms is performed with Progenesis QI software version 3.0 (Waters) and it is consisted of chromatogram alignment, peak detection, data normalization and PCA calculation. Compound annotation is done with MSFinder 3.54 software. Data processing of the raw GC-HRMS chromatograms is done with SureMass/Mass Profiler Professional which includes spectral deconvolution, combined with feature alignment across multiple data files and compound identification based on spectral database (NIST2020) matching.

3.4. Electrophysiological assays

The direct electrophysiological measurement of transmembrane ion currents is an effective method to study the effects of bioactive compounds (hits) on membrane-bound channels and receptors. The method that was applied to measure electrophysiological changes (i.e. fluxes of current) uses the *Xenopus laevis* oocyte expression system (Figure 4). The oocytes are isolated by partial ovariectomy from anesthetized *Xenopus laevis* frogs. cDNA present in the cDNA library of our laboratory is (or has been already) subcloned in a pGEMHE expression (HE = High Expression) vector, containing 3' and 5' untranslated regions (UTR) of a *Xenopus* β-globin gene. After in vitro transcription of the cDNA using a commercial T7 or SP6 mMESSAGE mMACHINE transcription kit (Ambion), injection of the *Xenopus* oocytes with the mRNA flanked by the UTR results in the very high expression of the respective ion channel or receptor. After in vivo translation, these ion channels or receptors will be correctly inserted in the cell membrane of the oocytes, and as such they can be studied in overexpression, devoid of contaminating pathways/other channels or receptors and guaranteeing the best possible pharmacological approach.

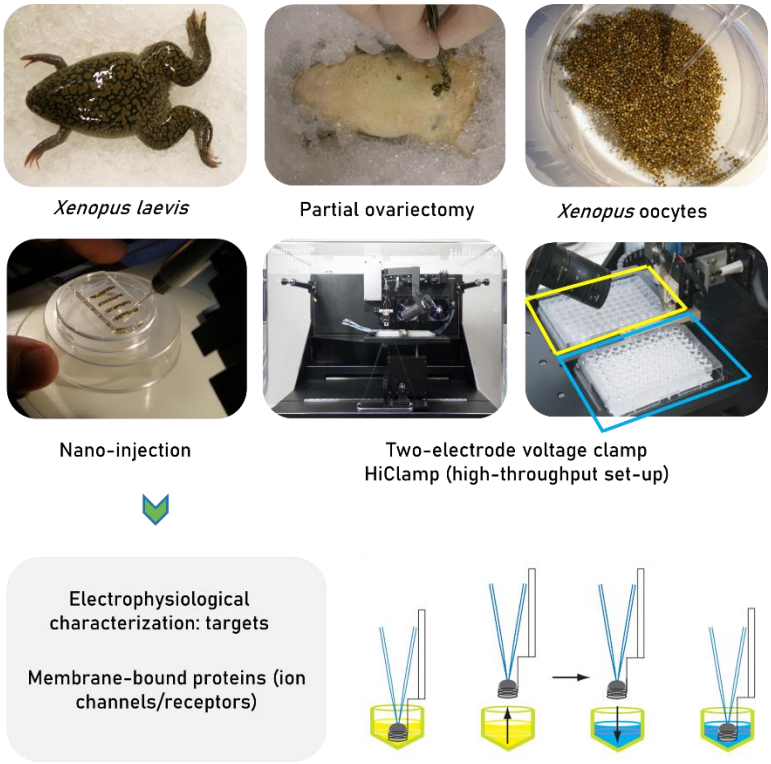


Figure 4: Experimental setup of electrophysiological assays.

The ionic current through the membrane was measured with the two-electrode voltage clamp technique (TEVC). With this technique the potential difference across the cell membrane can be controlled to observe current fluctuations. These fluctuations can directly be measured using a GeneClamp 500 amplifier (Axon Instruments) and/or HiClamp robot (Multi Channel Systems). The TEVC technique is ideal for compound screening because it is an open and robust bioassay system that both in depth characterisation of compounds, as well as high-throughput screening. The fluctuations in current give detailed and relevant information about the response of the ion channel or receptor to the compounds of interest.

From a pharmaceutical point of view, three molecular targets of interest in PROBIO: Kv1.3, nAChR and NAv1.4 were tested.

Kv1.3 is a potassium-selective, voltage-gated channel, also known as KCNA3, expressed as membrane-bound protein mainly in T and B lymphocytes. It is generally accepted that effective treatment of autoimmune and neuro-inflammatory diseases, such as multiple sclerosis (MS), stroke, epilepsy, Alzheimer's and Parkinson's disease, remains a challenge. Since Kv1.3 plays a crucial role in subsets of T and B lymphocytes, as well as microglial cells, it is considered a novel therapeutic target for treating these disorders (Figure 5). The discovery of a potent and selective blocker of Kv1.3 indeed may reveal a lead for a future therapeutic agent to treat autoimmune and neuro-inflammatory diseases.

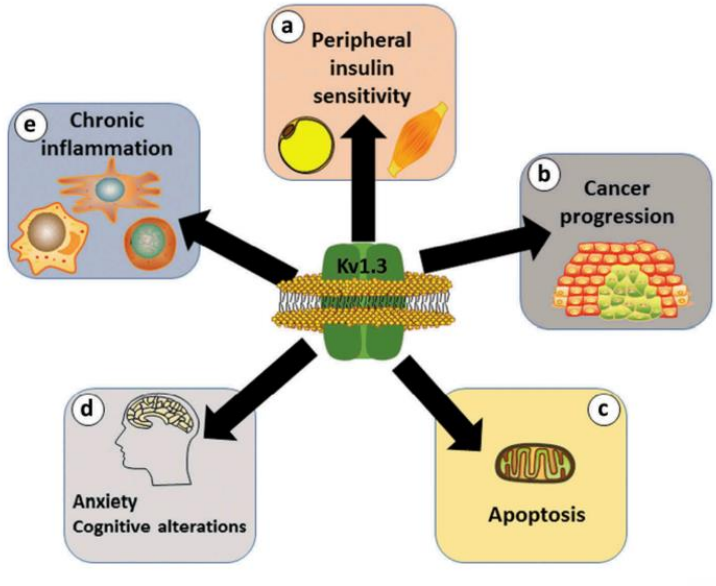


Figure 55: Several applications with regard to the voltage-gated potassium channel.

nAChR stands for nicotinic Acetylcholine Receptor, a membrane-bound peptide that respond to the neurotransmitter acetylcholine. Nicotinic receptors also respond to drugs such as the agonist nicotine. They are found in the central and peripheral nervous system, muscle, and as such play a crucial role in the cholinergic transmission in our body. They are involved in a complex range of central nervous system disorders including Alzheimer's and Parkinson's disease, schizophrenia, Tourette´s syndrome, anxiety, depression and epilepsy. Preventing the release of acetylcholine from axon endings at the neuromuscular junction or blocking the nAChR there, causes flaccid muscle paralysis and an anti-wrinkle effect. The discovery of a potent and selective blocker of nAChRs may reveal a lead for a future

therapeutic agent to treat central nervous system disorders, an anti-wrinkle cream and/or an anti-aging lotion successful in the world of cosmetics and personal facial care. Figure 6 illustrates the location and role of nicotinic receptors (nAChRs).

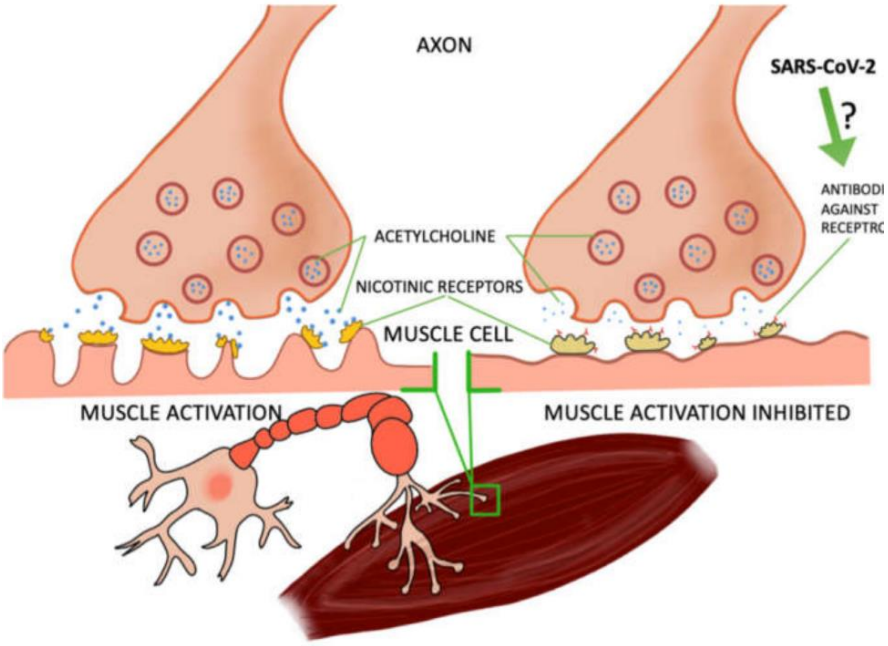


Figure 6: Location and role of nicotinic receptors (nAChRs).

NAv1.4. is a type of voltage-gated sodium channel, very important in skeletal muscle. Na currents flowing in the muscle cells through this channel will enable the muscle to contract (see Figure 7). Any ligand modulating this Nav1.4 channel therefore can be helpful in the treatment of muscle-related diseases, such as uncontrolled contractions and/or paralysis.

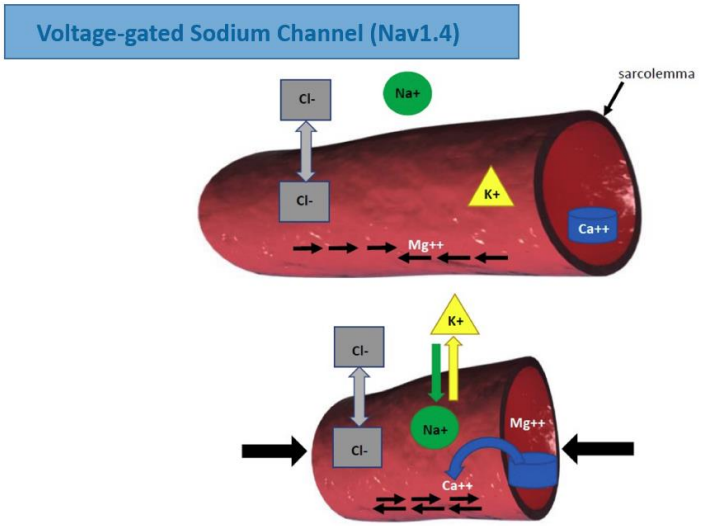


Figure 7: Illustration of the voltage-gated sodium channel.

Despite the high throughput set-up of the electrophysiological assays, the analysis is quite time-consuming. Since every sample (of the 19 samples in total) is divided into 96 fractions, a total of 1824 fractions per target needs to be tested. With a required time of 15min. per fraction, the time required to test all fractions is 27360 min. or approximately 5 months per target. Since the original screenings did not result in a fraction with convincing modulatory effect on the Nav1.4 target, and since efforts needed to be downscaled due to time constraints, the focus was put on the nAChR and the Kv1.3 channel.

3.5. Antimicrobial assays

The ability to interfere with microbial growth was assayed for a set of 'lead' extracts and/or compounds. Activity against planktonic (free-living) cells (first phase), as well as against biofilm associated cells (second phase) were evaluated.

In the first research phase, representatives of Gram-negative bacteria (*Pseudomonas aeruginosa, Acinetobacter baumannii*), Gram-positive bacteria (*Staphylococcus aureus*) and fungi (*Candida albicans*) were included to obtain a first insight into the spectrum of activity of the compounds. For determination of activity against planktonic cells, standardized methods also used in clinical microbiology were used. These broad micro-dilution methods allow us to determine the minimal inhibitory concentration (MIC, i.e. the lowest concentration that inhibits growth of the organism) and the minimal bactericidal/fungicidal concentration (MBC/MFC, i.e. the lowest concentration that results in complete killing of the organism). This work was carried out in 96-well microtiterplates (MTP) and the MIC was determined using absorbance measurements with an automated microtiter plate reader (Envision, Perkin Elmer) after 24h of incubation at 37°C. MBC/MFC determinations were based on results of plating (additional 24h of incubation). This approach guarantees a high throughput and an efficient workflow.

In the second research phase the antibiofilm activity of selected compounds (i.e. those compounds showing activity against planktonic cells) was evaluated using an MTP set-up. In this setup, organisms were added to the well of a MTP together with the bioactive (sub-)fraction (to determine biofilm-inhibitory activity) or the (sub)fraction was added after the organism is allowed to form a biofilm (to determine biofilm-eradicating activity). The anti-biofilm activity was determined using a culture-independent assay. To this end, after biofilm formation and/or exposure to extracts/compounds, resazurin was added to the wells of the MTP. Resazurin (also known as Alamar Blue, commercial name: CellTiterBlue, CTB) is a non-fluorescent compound that is converted to the highly fluorescent resorufin by living cells; the amount of fluorescence measured is directly related to the number of metabolically active (i.e. living) cells that are present in the biofilm.

4. Results

4.1. Bioprospection Index Cards

The species shortlist, resulting from the selection procedure described above, consists of 50 species of 13 different phyla occurring in the Belgian part of the North Sea (Figure 8).

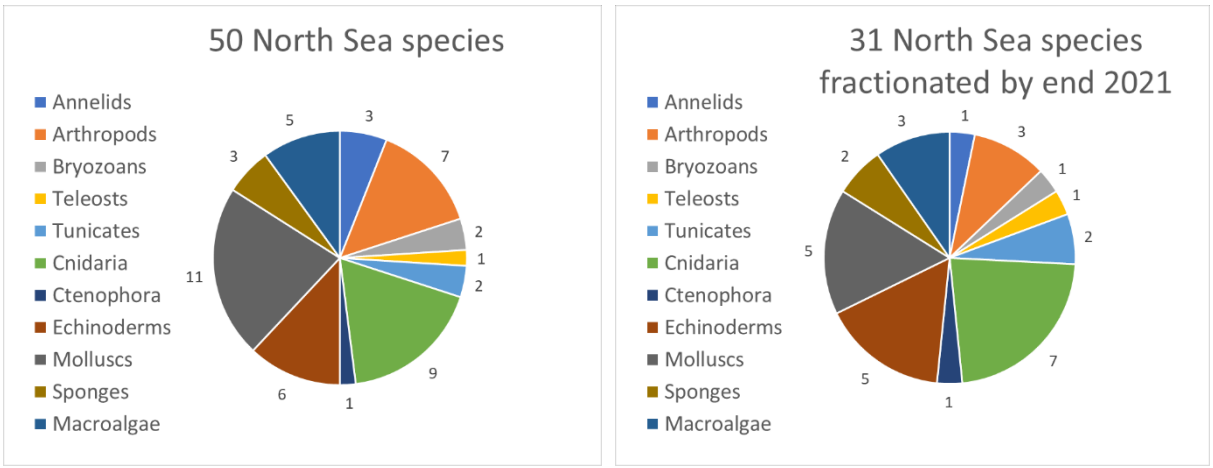


Figure 8: Distribution of North Sea species over the different phyla included in the PROBIO shortlist (left) and distribution of North Sea species that was further fractionated.

The Bioprospection Index Cards (BICs) (Figure 9) include a general description of the selected species together with an overview of spatial distribution, habitat and substrate preferences, proposed sampling methods and information on potential compounds and cultivation opportunities derived from a comprehensive literature study. The BICs gave input to all work packages within the PROBIO project, e.g. information for sample collection in WP1, knowledge on potential bioactive compounds for WP2, WP3 and WP4, and, input for the valorisation pathways in WP5. A first compilation was finalised in July 2020. An update for four species (*Asterias rubens, Ophiura albida, Ophiura ophiura, Sargassum muticum*) was included in the latest version, based on the increased interest for follow-up trajectories, as a result of the hits from the bioactivity assays. The Bioprospection Index Cards can be consulted in **SANDRA AND DE RAEDEMAECKER 2022** (Deliverable 1.3).

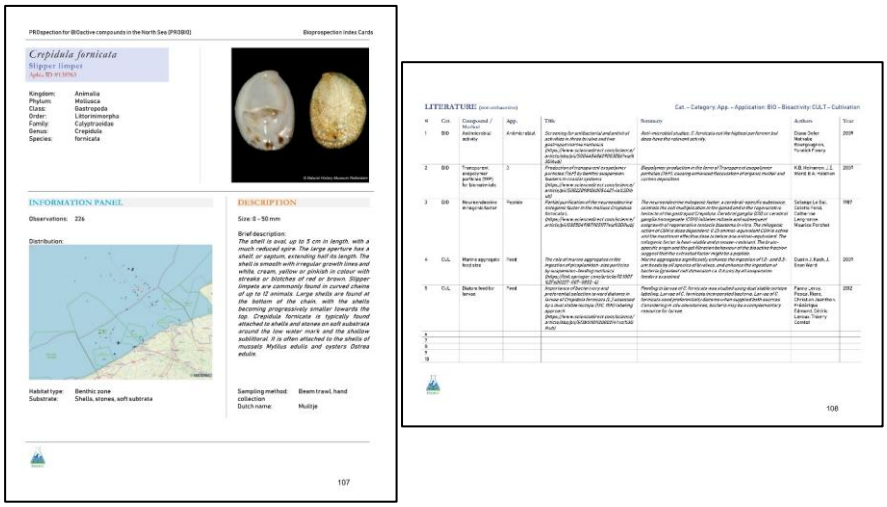


Figure 9: Exemplary Bioprospection Index Card of Crepidula fornicata.

4.2. Fractionation and subfractionation

A very concise and high-throughput protocol for fractionation of PROBIO samples was established and performed on 40 samples with high biodiversity and chemical diversity, covering a total of 31 species, and resulted in a total of 9888 fractions from marine organisms of the Belgian part of the North Sea (Table 1).

Table 1: Overview of fractionations per species and their effective analysis.

#	Species	Number of fractionated samples	Number of batches (WP2, 3 & 4)	Electrophysiological analysis	Antimicrobial analysis
1	*Alcyonium digitatum*	1	3	Yes	Yes
2	*Alloteuthis subulata*	1	2	No	Yes
3	*Asterias rubens*	3 (1 trial sample)	9	Yes	Yes
4	*Aurelia aurita*	1	2	No	Yes
5	*Botrylloides violaceus*	1	2	No	Yes
6	*Chrysaora hysoscella*	2	6	Yes	Yes
7	*Codium fragile*	1	2	No	Yes
8	*Crangon crangon*	2	6	Yes	Yes
9	*Crepidula fornicata*	1	2	No	Yes
10	*Cyanea lamarckii*	1	3	Yes	Yes
11	*Echiichthys vipera*	2	6	Yes	Yes
12	*Echinocardium cordatum*	2	6	Yes	Yes
13	*Ectopleura larynx*	1	3	Yes	Yes
14	*Ensis spp.*	1	2	No	Yes
15	*Halichondria panicea*	1	2	No	Yes
16	*Haliclona oculata*	1	2	No	Yes
17	*Lanice conchilega*	1	2	No	Yes
18	*Liocarcinus depurator*	1	2	No	Yes
19	*Liocarcinus holsatus*	2	4	Partly	Yes
20	*Metridium senile*	1	3	Yes	Yes
21	*Mytilus edulis*	2 (1 trial sample)	6	Yes	Yes
22	*Ophiura albida*	1	2	No	Yes
23	*Ophiura ophiura*	2	6	Yes	Yes
24	*Pleurobrachia pileus*	1	2	No	Yes
25	*Psammechinus miliaris*	1	2	Partly	Yes
26	*Sagartia spp.*	1	2	No	Yes
27	*Sargassum muticum*	1	2	No	Yes
28	*Styela clava*	1	2	No	Yes
29	*Tricellaria inopinata*	1	2	No	Yes
30	*Tritia reticulata*	1	2	No	Yes
31	*Ulva pseudocurvata*	1	2	No	Yes

The 40 samples fractionated, included 2 trial samples harvested in March 2020 while 38 samples belonged to the final list of samples covering a total of 31 species (Table 1 and Deliverable 2.2). For the first 21 samples fractionated (incl. 2 trial samples), three similar fractionations were performed resulting in three batches of 96 fractions, two batches were sent to WP3 and WP4 for biological screening, while one batch of fractions was stored at MCG-VIB for analytical screening. For the other 20 samples

fractionated, only two similar fractionations were performed resulting in two batches of 96 fractions that were sent to WP4 and one batch stored at VIB-MCG. Due to the complexity of the workflow of the bioassays in WP3, which resulted in a delay in processing fractions produced by MCG-VIB, it was decided that for the last 20 samples only two similar fractionations would be generated, one batch for WP4 and one batch stored at MCG-VIB (Table 1).

The selection of the fractions (from specific species) that were further subfractionated was based on the results from WP3 and WP4 (see section 4.5 & 4.6). In Table 2 an overview can be found of subfractionated positive preparative fractions (including number of subfractions) and the corresponding species. The table shows how many injections were done for each preparative fraction, and how many subfractions were generated based on the complexity of the respective fraction.

Table 2: Selected preparative fractions (found positive for containing bioactivity) for subfractionation and number of sub-fractions generated for WP 3 (green) and WP4 (blue).

Species	Common name	Season	prep fraction	# sub-fractions	prep fraction	# sub-fractions
Alcyonium digitatum	Dead man's finger	September	F4	45	F5	46
Echiichthys vipera	Lesser weever	June	F2	38		
Ophiura albida	Serpent's table brittle star	combined	F55	92	F83	43
Ophiura ophiura	Serpent brittle star	June	F55	71	F83	43
Ophiura ophiura	Serpent brittle star	combined	F65	94		
Liocarcinus depurator	Harbour crab	combined	F80	95		
Asterias rubens	Common starfish	March/June/September	F81	85		

4.3. Analytical screening

The analytical screening reported here consisted of 374 samples (50 species, different locations, different seasons) that were profiled. For each sample three profiles (UPLC-HRMS negative and positive ionization mode and GC-HRMS) were produced resulting in a total of 1122 analyses/chromatograms.

4.3.1. UPLC-HRMS results

All UPLC-HRMS profiles were processed through Progenesis QI software. Due to the different nature of the samples no alignment was applied prior to peak picking. Peak picking included the following processes: run aggregation, compound ion detection, isotope deconvolution, adduct deconvolution, quantitation and normalization. This resulted in a number of picked compound ions representing the whole set of samples. For peak picking purposes the following parameters were used:

- Runs to include: all;
- Sensitivity: 3 (default);
- Minimum peak width: 0.05 minutes.

For negative ionization mode the peak picking resulted in 21847 compound ions, for positive ionization we detected 22624 compound ions. To visualize general differences within the different phyla, Principal Component Analysis (PCA) was performed on all compound ions picked for each ionization mode to reduce the high number of features and increase the interpretability of the metabolic differences or similarity between phyla. Figures 10-11 show PCA plots per phylum for negative and positive ionization mode, respectively. Here a cut-off was used for compounds that have at least 500 counts (signal abundance) and PCA's were based on the top 1000 most differential compounds based on ANOVA.

Based on these cut-offs, it becomes more visible which phyla are more or less closely related to each other based on their metabolic content. It also shows the variance of metabolites within a phylum, as a result of different abundances between different seasons and/or locations.

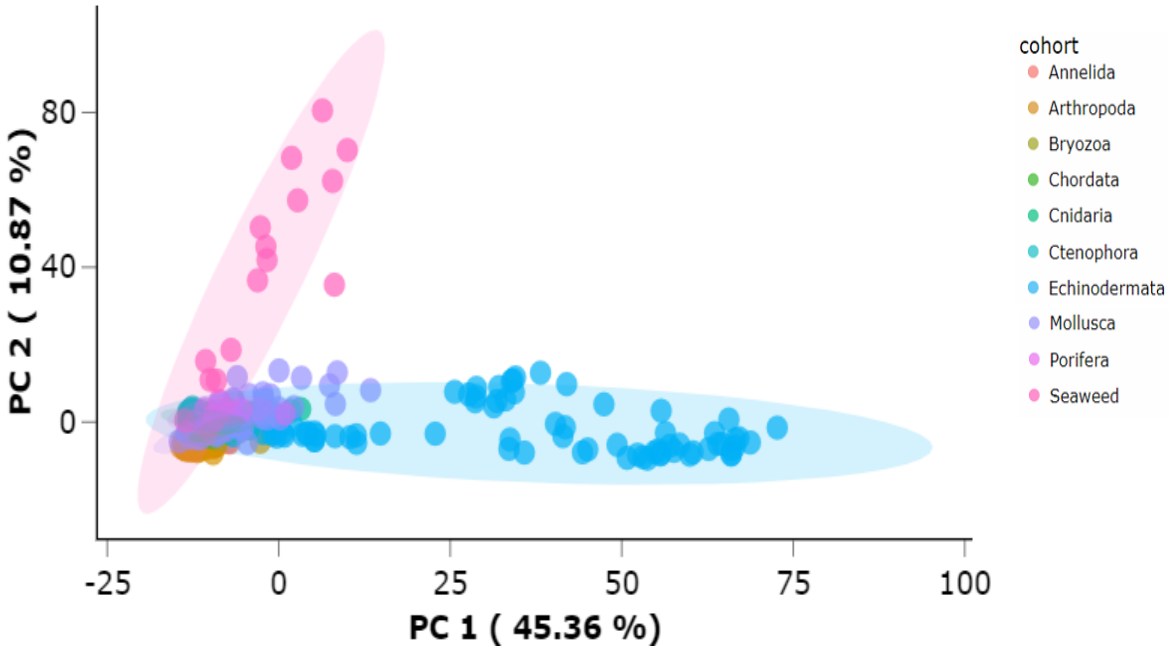


Figure 10: PCA plot per phylum generated from the 6150 compound ions (> 500 counts & 1000 most significant differentials based on ANOVA) form the LC-MS positive ionization mode showing moderate grouping of the different phyla based on their metabolic content.

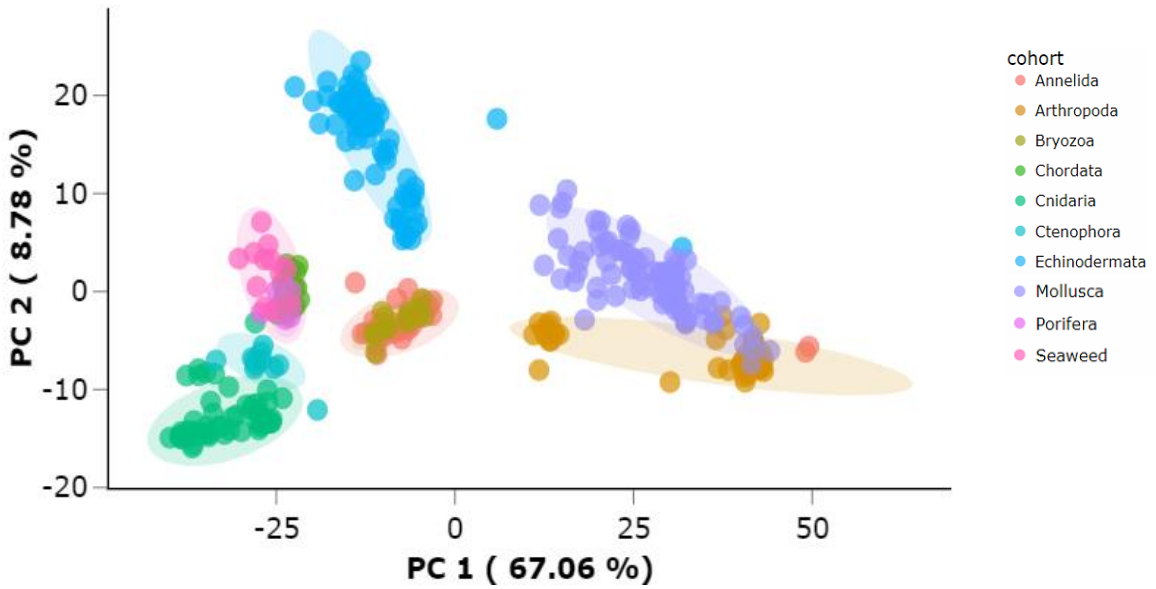


Figure 11: PCA plot per phylum generated from the 2334 compound ions (> 500 counts & 1000 most significant differentials based on ANOVA) form the LC-MS negative ionization mode showing clear grouping of the different phyla based on their metabolic content.

For compound annotation/identification purposes, the exact mass, isotopic pattern and fragmentation data of each compound ion was used for matching against online available spectral libraries for natural products through the open access MSFinder 3.54 software. Results for annotations can be found in the heatmap (section 4.4) available at the VLIZ website (GOEMINNE ET AL., 2022A). These annotations are the best matching (based on spectral similarity) hits that are available in public spectral databases, but are not absolute identifications.

4.3.2. GC-HRMS results

GC-HRMS analysis resulted in 9359 compound signals. To visualize the general differences within the project a Principal Component Analyses (PCA) was performed on all compound ions. Figure 12 shows the PCA plot per phylum for the GC-HRMS analysis.

For identification purposes, the GC-MS chromatograms were deconvoluted using Analyzer Pro, and compounds corresponding to the deconvoluted spectra were matched (using a match factor of 70% as cut-off) against the NIST 20 mass spectral library. The heatmap for GC-MS is available at the VLIZ website (GOEMINNE ET AL., 2022B).

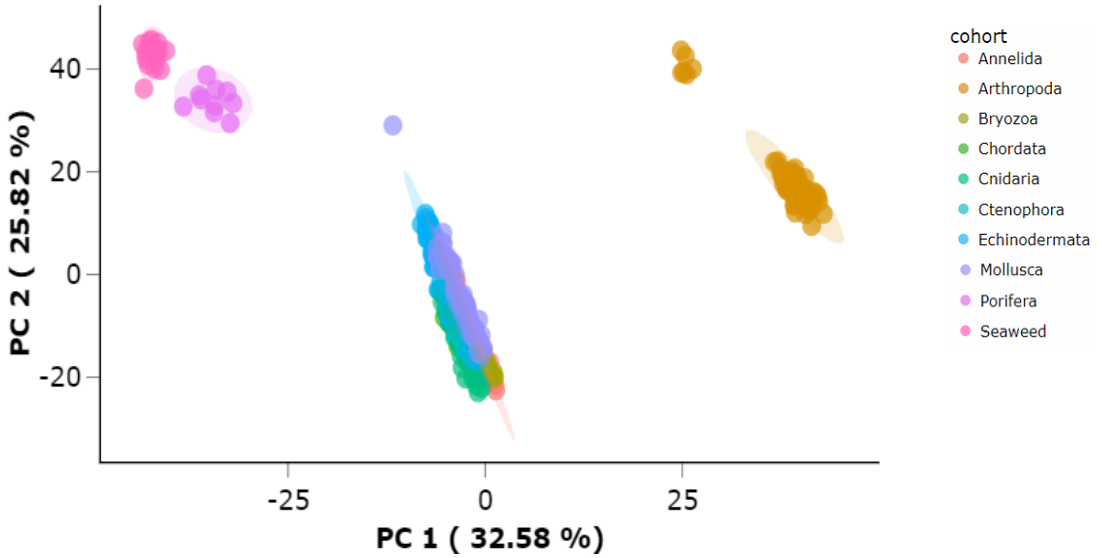


Figure 12: PCA plot per phylum generated from the 9359 compound signals (1000 most significant differentials based on ANOVA) present on the GC-HRMS profiles.

4.3.3. Conclusions of the analytical screening

The analytical screening resulted in a highly valuable dataset about 50 different species from the Belgian part of the North Sea, covering a total of 10 phyla. In total more than 20K compound ions (for LC-MS) and +9K features (for GC-MS) were measured for this set of samples. For each compound ion a set of information is available: its accurate mass, ion mobility data (for LC-MS only), fragmentation pattern, and its abundance/occurrence through species, phylum, geographical location and season. Raw data was converted to an open source format (mzML) and can be requested by sending an email to

geert.goeminne@vib.be. A link will then be provided giving access to download the data. The processed data is downloadable at the VLIZ website for both LC-MS and GC-MS (GOEMINNE ET AL., 2022A; GOEMINNE ET AL., 2022B) (see section 4.4). PCA shows clear differences between phyla but also shows which phyla are more closely related to each other based on their metabolic content. PCA on subsets (specific phyla, different locations or seasons) can be performed on the processed data available at VLIZ for more details on the metabolic similarity/variability within phyla. All metadata on location and season is available for each sample.

4.4. Marine Compounds Database

4.4.1. Raw data

All HR GC- and LC-MS raw data (open source mzML format) resulting from the analytical screening are available for the Scientific Community and can be requested as described in section 4.3.3.
This dataset contains all following analyses:

- GC-HRMS of all samples (374 EI chromatograms)
- UPLC-HRMS in positive ionization of all samples (374 ESI+ chromatograms)
- UPLC-HRMS in negative ionization of all samples (374 ESI- chromatograms)

Each dataset contains all compound ions that were detected (including retention time, accurate mass and abundance) for each marine organism, each season and each location. Open source tools such as MZMINE 3.0 can be used to upload, view and process the raw data files.

4.4.2. Processed data

For LC-MS, all compound ions with a reasonable abundance (>500 counts) were matched against online (spectral and chemical) databases for putative annotation of already known compounds (dereplication), and are represented in the heatmap summary as a top 10 of the best matching structure (including a score from 0 to 10 for the spectral or in-silico fragmentation match). For GC-MS, data was matched against NIST20 database, the golden standard library for GC-MS. These datasets are available as .JSON-files and can be downloaded from the VLIZ website (LC-MS: GOEMINNE ET AL., 2022A; GC-MS: GOEMINNE ET AL., 2022B). As visualization tool we recommend to use the PHANTASUS TOOL for data visualisation. Several search options are available (search for species, season, phylum, m/z, compound name, …) and filtering options are available as well to select for subsets. The abundance of each compound ion is visualized in the form of a heatmap (Figure 13 and 14), and for each selected compound ion a chart (line or boxplot) can be generated from within the application showing the variance within a certain cohort, phylum or through the whole dataset (for each sample) (Figure 15). The whole dataset including all potential annotations and other information can also be exported to .csv or excel format to allow the scientific community to use the data through other applications.

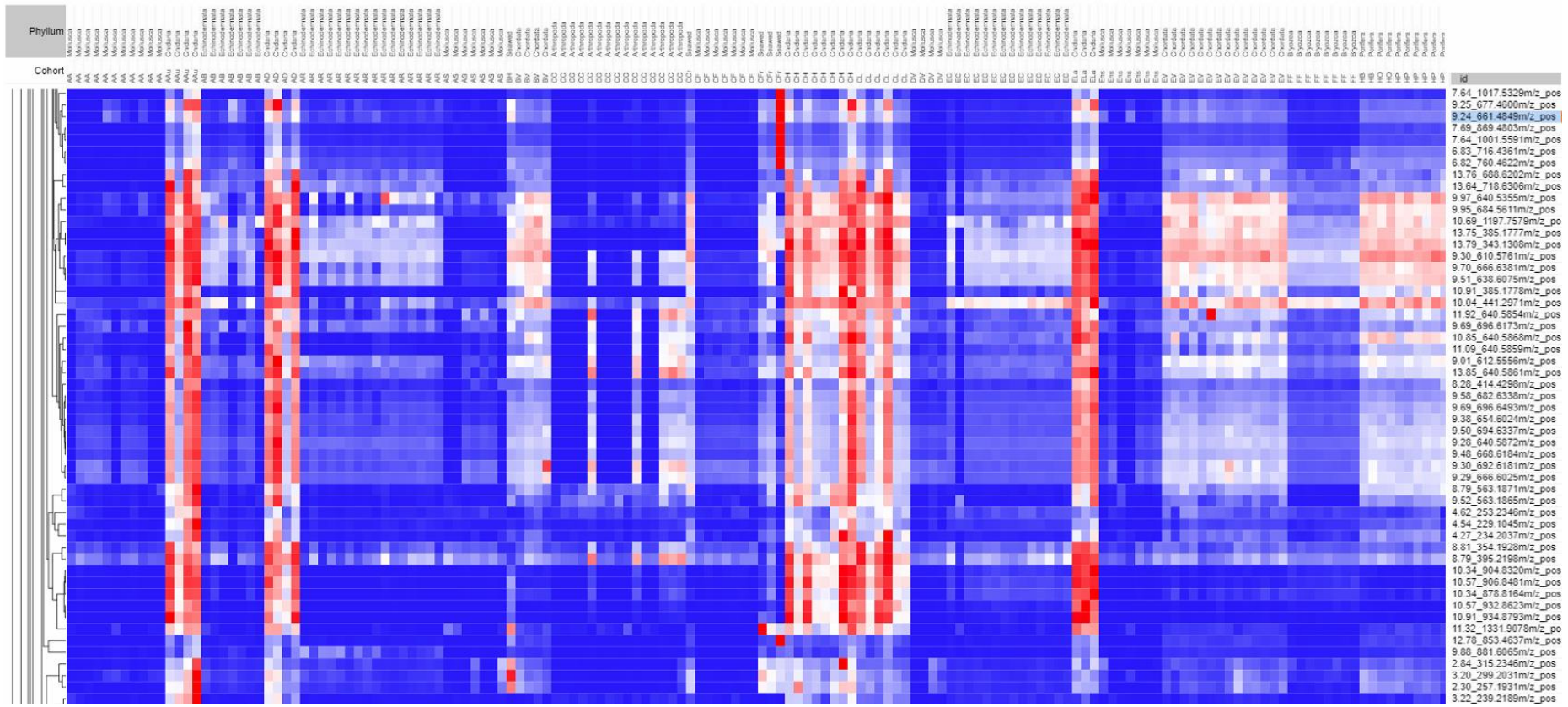
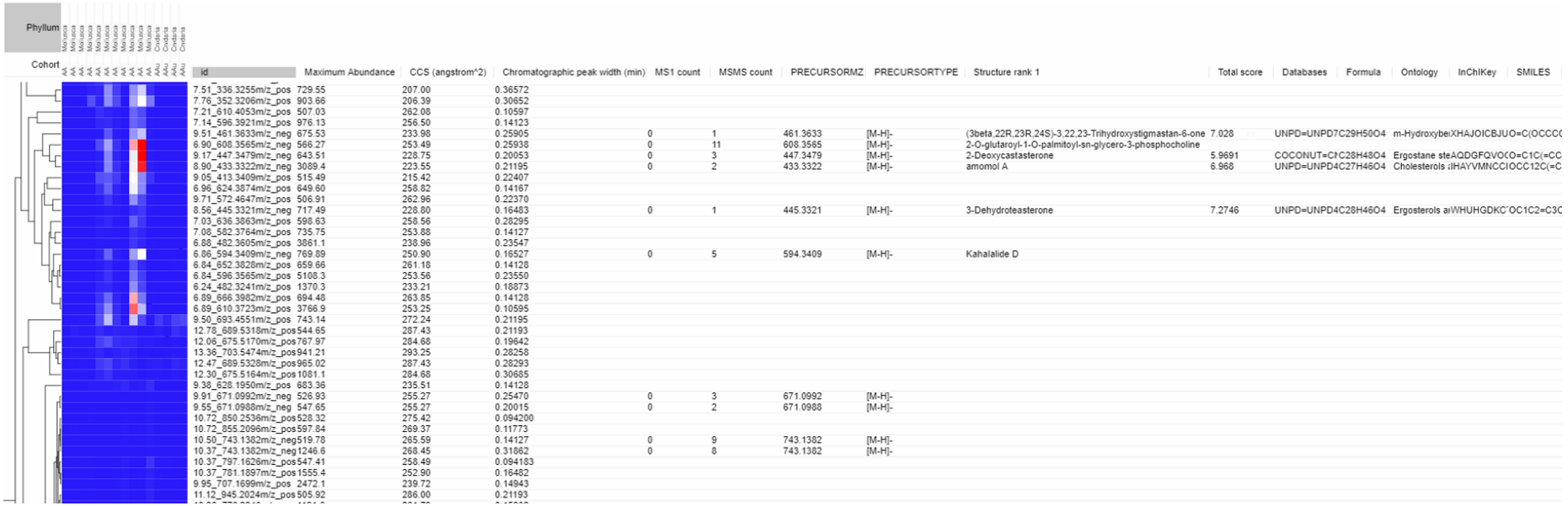


Figure 13: Heatmap representation of all samples (horizontal axis on TOP, including cohort and phylum names) and all detected compound ions (vertical axis) represented by their Rt_m/z. The more red, the higher the abundance of a compound is, the more blue, the lower abundant.



id	Maximum Abundance	CCS (angstrom^2)	Chromatographic peak width (min)	MS1 count	MSMS count	PRECURSORMZ	PRECURSORTYPE	Structure rank 1	Total score	Databases	Formula	Ontology	InChIKey	SMILES
7.51_336.3255m/z_pos	729.55	207.00	0.36572											
7.76_352.3206m/z_pos	903.66	206.39	0.30652											
7.21_610.4053m/z_pos	507.03	262.08	0.10597											
7.14_596.3921m/z_pos	976.13	256.50	0.14123											
9.51_461.3633m/z_neg	675.53	233.98	0.25905	0	1	461.3633	[M-H]-	(3beta,22R,23R,24S)-3,22,23-Trihydroxystigmastan-6-one	7.028	UNPD=UNPD7C29H50O4	m-HydroxybeiXHAJOICBJUO=C(OCCC(			
6.90_608.3565m/z_neg	566.27	253.49	0.25938	0	11	608.3565	[M-H]-	2-O-glutaroyl-1-O-palmitoyl-sn-glycero-3-phosphocholine						
9.17_447.3479m/z_neg	643.51	228.75	0.20053	0	3	447.3479	[M-H]-	2-Deoxycastasterone	5.9691	COCONUT=CNC28H48O4	Ergostane steAQDGFQVOCO=C1C(=CC			
8.90_433.3322m/z_neg	3089.4	223.55	0.21195	0	2	433.3322	[M-H]-	amomol A	6.968	UNPD=UNPD4C27H46O4	Cholesterols alHAYVMNCCIOCC12C(=C			
9.05_413.3409m/z_pos	515.49	215.42	0.22407											
6.96_624.3874m/z_pos	649.60	258.82	0.14167											
9.71_572.4647m/z_pos	506.91	262.96	0.22370											
8.56_445.3321m/z_neg	717.49	228.80	0.16483	0	1	445.3321	[M-H]-	3-Dehydroteasterone	7.2746	UNPD=UNPD4C28H46O4	Ergosterols aiWHUHGDKC'OC1C2=C3C			
7.03_636.3863m/z_pos	598.63	258.56	0.28295											
7.08_582.3764m/z_pos	735.75	253.88	0.14127											
6.88_482.3605m/z_pos	3861.1	238.96	0.23547											
6.86_594.3409m/z_neg	769.89	250.90	0.16527	0	5	594.3409	[M-H]-	Kahalalide D						
6.84_652.3828m/z_pos	659.66	261.18	0.14128											
6.84_596.3565m/z_pos	5108.3	253.56	0.23550											
6.24_482.3241m/z_pos	1370.3	233.21	0.18873											
6.89_666.3982m/z_pos	694.48	263.85	0.14128											
6.89_610.3723m/z_pos	3766.9	253.25	0.10595											
9.50_693.4551m/z_pos	743.14	272.24	0.21195											
12.78_689.5318m/z_pos	544.65	287.43	0.21193											
12.06_675.5170m/z_pos	767.97	284.68	0.19642											
13.36_703.5474m/z_pos	941.21	293.25	0.28258											
12.47_689.5328m/z_pos	965.02	287.43	0.28293											
12.30_675.5164m/z_pos	1081.1	284.68	0.30685											
9.38_628.1950m/z_pos	683.36	235.51	0.14128											
9.91_671.0992m/z_neg	526.93	255.27	0.25470	0	3	671.0992	[M-H]-							
9.55_671.0988m/z_neg	547.65	255.27	0.20015	0	2	671.0988	[M-H]-							
10.72_850.2536m/z_pos	528.32	275.42	0.094200											
10.72_855.2096m/z_pos	597.84	269.37	0.11773											
10.50_743.1382m/z_neg	519.78	265.59	0.14127	0	9	743.1382	[M-H]-							
10.37_743.1382m/z_neg	1246.6	268.45	0.31862	0	8	743.1382	[M-H]-							
10.37_797.1626m/z_pos	547.41	258.49	0.094183											
10.37_781.1897m/z_pos	1555.4	252.90	0.16482											
9.95_707.1699m/z_pos	2472.1	239.72	0.14943											
11.12_945.2024m/z_pos	505.92	286.00	0.21193											

Figure 14: Additional information (such as CCS values, structure rank 1,2,3… for potential annotation candidates, matching scores, database found, ontology, SMILES, …) can be found in the columns on the right, for each compound ion. Annotations and scores are based on spectral database matching and in-silico fragmentation described in section 4.3.1.

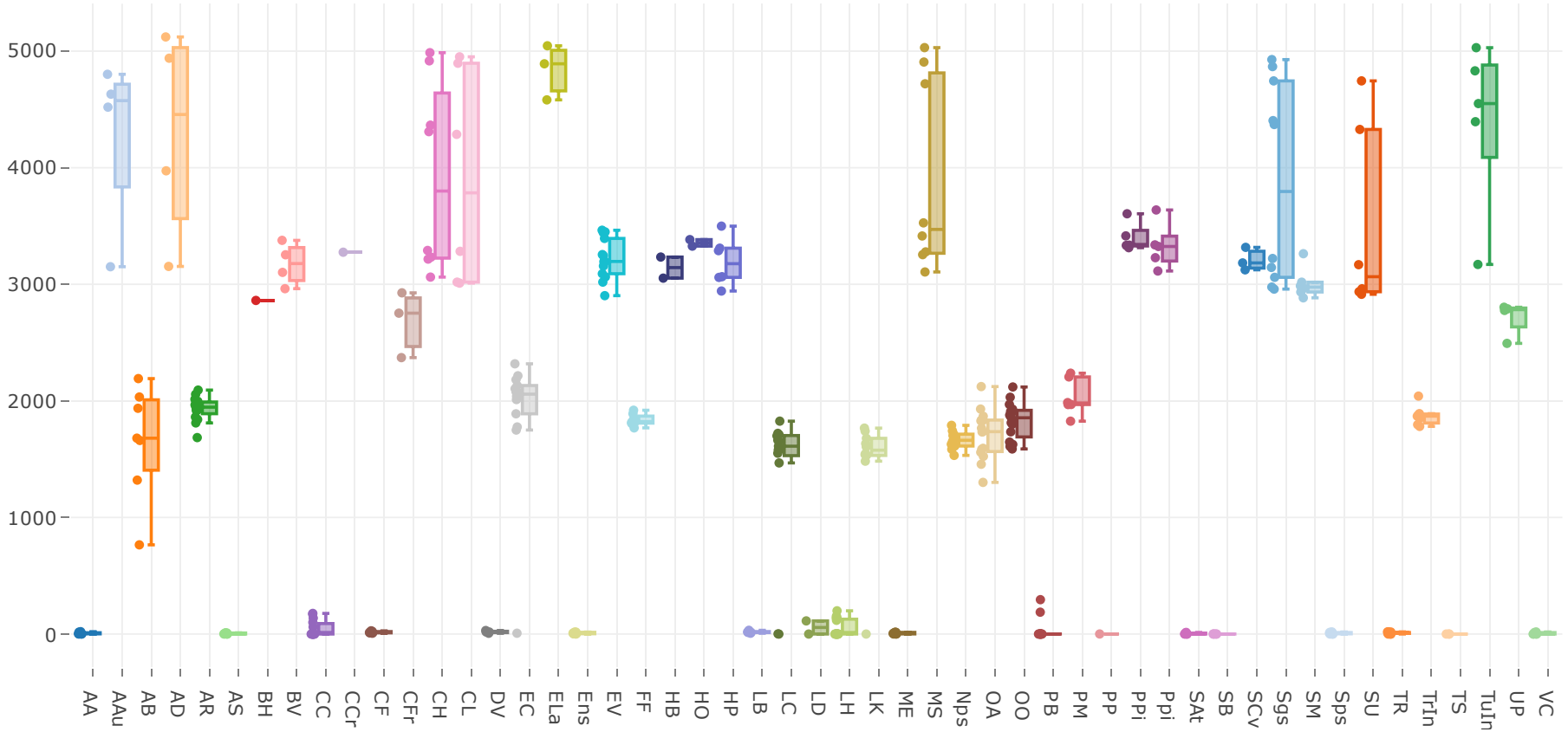


Figure 15: Example for unknown compound 13.79_343.1308m/z pos in all cohorts (X-axis) showing the abundance (Y-axis) of the compound in all samples where it was detected (LC-MS ESI positive mode).

4.5. Electrophysiological assays

Electrophysiological assays were carried out for 19 samples of 13 species (Table 1). Due to limited resources, not all of the promising fractions presented in the tables below could be subfractionated. Only two fractions from *Asterias rubens* (F81) and *Ophiura ophiura* (F65) were subfractionated (Table 2) but due constraints in resources, electrophysiological assays on subfractions have not been carried out in WP3.

4.5.1. nAChR channel

Partial or complete blockage of the nAChR channel was detected for several fractions of several samples. A summary of those fractions with a blockage equal to or exceeding 80% is given in Table 3. Deliverable 3.1 with the lists of all fractions per sample can be consulted for a full overview of the results/partial blockages per fraction of all samples tested.

Table 3: 'Hit' fractions showing a blockage of the nAChR channel for at least 80%, with an indication of the specific species and the season of the samples.

Target	Species	Common name	Season	Fraction	Effect
nAChR (adult)	*Asterias rubens*	Common starfish	March	F71	80% Block
nAChR (adult)	*Asterias rubens*	Common starfish	March	F81	90% Block
nAChR (adult)	*Asterias rubens*	Common starfish	June	F70-F72	90% Block
nAChR (adult)	*Asterias rubens*	Common starfish	June	F81-F82	80-100% Block
nAChR (adult)	*Asterias rubens*	Common starfish	September	F74-F82	80-100% Block
nAChR (adult)	*Asterias rubens*	Common starfish	September	F84-F85	85-90% Block
nAChR (adult)	*Chrysaora hysoscella*	Compass jellyfish	June	F76-F90	100% Block
nAChR (adult)	*Crangon crangon*	Common shrimp	September	F41-F45	100% Block
nAChR (adult)	*Crangon crangon*	Common shrimp	September	F51-F55	100% Block
nAChR (adult)	*Crangon crangon*	Common shrimp	September	F71-F90	100% Block
nAChR (adult)	*Cyanea lamarckii*	Blue jellyfish	June	F76-F85	100% Block
nAChR (adult)	*Ectopleura larynx*	Ringed tubularian	March	F77-F84	80-100% Block
nAChR (adult)	*Echiichthys vipera*	Lesser weever	June	F80-F82	90% Block
nAChR (adult)	*Liocarcinus holsatus*	Harbour crab	June	F61-F90	100% Block
nAChR (adult)	*Ophiura ophiura*	Serpent brittle star	June	F41-F45	20-95% Block
nAChR (adult)	*Ophiura ophiura*	Serpent brittle star	June	F50-F54	75-90% Block
nAChR (adult)	*Ophiura ophiura*	Serpent brittle star	June	F66-F83	20-90% Block
nAChR (adult)	*Psammechinus miliaris*	Green sea urchin	June	F86-F90	100% Block

A typical example is shown in Figure 16 showing fraction 81 from *Asterias rubens*, which is able to fully block the inward cationic currents through the nACh-receptor. Upon wash-out, the effect is reversible.

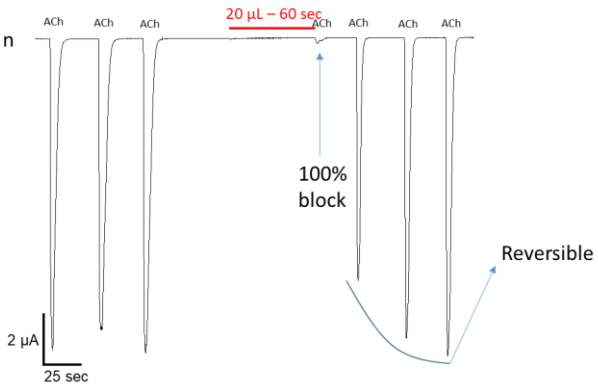


Figure 16: Reversible inhibitory effect of fraction 81 from Asterias rubens. fully blocking the inward cationic currents through the nACh-receptor.

4.5.2. Kv1.3 channel

Partial or complete blockage of the Kv1.3 channel was detected for several fractions of several samples. A summary of those fractions with all blockages is given in Table 4. Deliverable 3.1 with the lists of all fractions per sample can be consulted for a full overview of the results/partial blockages per fraction of all samples tested.

Table 4: 'Hit' fractions showing a blockage of the Kv1.3 channel, with an indication of the specific species and the season of the samples.

Target	Species	Common name	Season	Fraction	Effect
Kv1.3	*Alcyonium digitatum*	Dead man's finger	September	F1-F15	Block
Kv1.3	*Alcyonium digitatum*	Dead man's finger	September	F31-F35	Block
Kv1.3	*Alcyonium digitatum*	Dead man's finger	September	F56-F65	Block
Kv1.3	*Alcyonium digitatum*	Dead man's finger	September	F76-80	Block
Kv1.3	*Asterias rubens*	Common starfish	March	F71-F74	10%-20% Block
Kv1.3	*Chrysaora hysoscella*	Compass jellyfish	June	F11-F20	Block
Kv1.3	*Chrysaora hysoscella*	Compass jellyfish	June	F26-F30	Block
Kv1.3	*Chrysaora hysoscella*	Compass jellyfish	June	F41-F45	Block
Kv1.3	*Chrysaora hysoscella*	Compass jellyfish	June	F56-F65	Block
Kv1.3	*Chrysaora hysoscella*	Compass jellyfish	June	F81-F90	Block
Kv1.3	*Crangon crangon*	Common shrimp	September	F11-F35	Block
Kv1.3	*Crangon crangon*	Common shrimp	September	F41-F55	Block
Kv1.3	*Crangon crangon*	Common shrimp	September	F66-F96	Block
Kv1.3	*Cyanea lamarckii*	Blue jellyfish	June	F76-F90	Block
Kv1.3	*Ectopleura larynx*	Ringed tubularian	March	F81-F82	10%-50% Block
Kv1.3	Echiichthys vipera	Lesser weever	June	F80-F82	40% - 50% Block
Kv1.3	*Liocarcinus holsatus*	Harbour crab	June	F71-F75	Block
Kv1.3	*Ophiura ophiura*	Serpent brittle star	June	F34-F78	20-100% Block
Kv1.3	*Ophiura ophiura*	Serpent brittle star	June	F63-F65	100% Block
Kv1.3	*Psammechinus miliaris*	Green sea urchin	June	F36-F40	Block
Kv1.3	*Psammechinus miliaris*	Green sea urchin	June	F51-F96	Block

As illustrated in Figure 17, fraction 65 from *Ophiura ophiura* fully inhibits Kv1.3 and the effect is completely reversible after a period of 30s wash out.

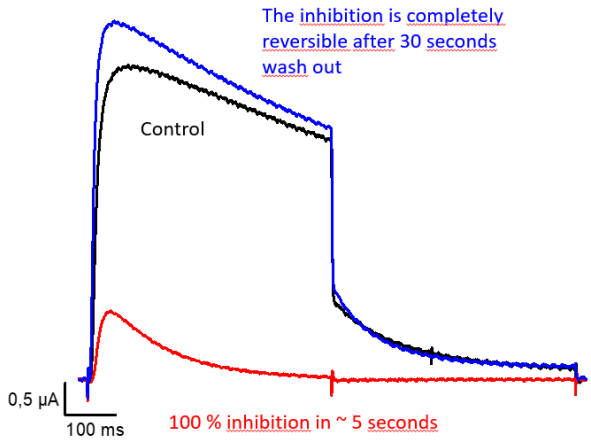


Figure 17: Reversible inhibitory effect of fraction 65 of Ophiura ophiura. fully blocking the inward cationic currents through the Kv1.3-receptor.

4.5.3. Nav1.4 channel

As introduced in section '3.4 Electrophysiological assays', the assays on the Nav1.4 channel was tested for a limited number of samples. This was reflected by the limited number of fractions and samples for which a partial blockage of the Nav1.4 channel was detected. A summary of those fractions with all blockages is given in Table 5. A small effect of 10% only was also included since there were very limited fractions showing an effect. Deliverable 3.1 with the lists of all fractions per sample can be consulted for a full overview of the results/partial blockages per fraction of all samples tested.

Table 5: 'Hit' fractions showing a blockage of the nAChR channel for at least 80%, with an indication of the specific species and the season of the samples.

Target	Species	Common name	Season	Fraction	Effect
Nav1.4	*Echiichthys vipera*	Lesser weever	*June*	F40	10% Block
Nav1.4	*Ophiura ophiura*	Serpent brittle star	*June*	F66-F69	40%-70% Block

4.5.4. Conclusions of the electrophysiological assays

Electrophysiological assays were carried out for 19 samples of 13 species. From a set of targets (ion channels or receptors) that are associated with certain physiological processes in humans and animals, 3 targets were selected for this project: adult muscle type nicotinic acetylcholine receptor (nAChR), voltage-gated muscle type sodium channel (Nav1.4) and voltage-gated potassium channel (Kv1.3), after advice from the Strategic Advisory Board thus aiming at a broad valorisation. The active effect of the metabolites on these targets is measured as potential difference in oocytes of the model organism *Xenopus laevis*. Partial or complete blockage of the nAChR channel and the Kv1.3 channel was detected for several fractions of several samples. The assays on the Nav1.4 channel were carried out for a limited number of samples, also reflecting the limited number of fractions and samples for which a partial blockage of the Nav1.4 channel was detected. Based on the results, two positive fractions from *Asterias rubens* (F81) and *Ophiura ophiura* (F65) were subfractionated for further bio-activity testing in WP3. However, due to limited resources, assays on subfractions have not been carried out in WP3. The results of the fractions from *O. ophiura* are similar to the results of *A. rubens*, and since these two organisms are closely related on the phylogenetic tree it is plausible that they have common bioactive molecules (e.g. saponins). Saponins are molecular structures that are known to have anti-bacterial and anti-viral properties. These species use saponins as a defense mechanism against predators and diseases, which make these compounds a very interesting subject to study for valorisation.

4.6. Antimicrobial assays

Antimicrobial assays were carried out for 31 species (see Table 1).

4.6.1. Growth inhibitory activity towards planktonic cells

Antimicrobial activity was detected in a wide range of organisms and fractions. A full overview of the results obtained from the initial screening of growth inhibitory activity towards planktonic cells of *Staphylococcus aureus, Acinetobacter baumannii, Pseudomonas aeruginosa and Candida albicans* is shown in S-table 1 (in Supplementary tables). Only marine organisms and fractions that showed a hit against at least one of the test organisms are included.

Antimicrobial activity was particularly present in 'early' (F2-F4) and 'late' (F80-F85) fractions. This pattern occurred in a wide taxonomic distribution suggesting that antimicrobial activity in these 'early' and 'late' fractions is aspecific, and possibly attributable to high salt concentration ('early' fractions) and the presence of surfactants ('late' fractions).

Excluding the results from F2-6 and F80-88, other interesting fractions were identified with pronounced activity against the test organisms (Table 6).

Table 6: Selection of the results obtained from the screening of growth inhibitory activity towards planktonic microorganisms. Only marine organisms and fractions that showed a hit against at least one of the test organisms is shown with the exclusion from 'early' (F2-F6) and 'late' (F80-F85) fractions.

Planktonic microorganism	Species	Common name	Month	Fraction
Staphylococcus aureus	*Asterias rubens*	Common starfish	June, September	F48, F50, F59
	Echinocardium cordatum	Sea potato	June, September	F45, F46, F51
	Ophiura albida	Serpent's table brittle star	pooled	F38-59
	Ophiura ophiura	Serpent brittle star	June, September	F38-F64
	Sargassum muticum	Strangleweed	pooled	F8-F20
Acinetobacter baumannii	*Lanice conchilega*	*Sand mason worm*	pooled	F16, F17
Pseudomonas aeruginosa	*Lanice conchilega*	*Sand mason worm*	pooled	F16, F17
Candida albicans	*Mytilus edulis*	Blue mussel	pooled	F15

4.6.2. Killing activity towards planktonic cells

For a selection of the fractions with a high growth-inhibitory activity, killing activity was assessed. The results are summarized in S- table 2 (bactericidal activity of selected fractions against *S. aureus*), S-table 3 (bactericidal activity of selected fractions against *A. baumannii* and *P. aeruginosa*) and S-table 4 (bactericidal activity of selected fractions against *C. albicans*).

A summary of the organisms and fractions that showed killing activity against at least one of the test organisms is presented in Table 7. A more detailed specification on the incubation time allowed (24h or 48h) to kill off the organisms is given in S-Tables 2-4.

Table 7: Selection of the results obtained from the screening of killing activity towards planktonic organisms. Only marine organisms and fractions that showed killing activity against at least one of the test organisms is shown. A complete overview of the activity for all fractions tested is given in S-Table 2-4.

Planktonic microorganism	Species	Common name	Month	Fraction
Staphylococcus aureus	*Asterias rubens*	Common starfish	June	F83
	Crangon crangon	Common shrimp	December	F83 - F86
	Echiichthys vipera	Lesser weever	June	F2
	Ophiura albida	Serpent's table brittle star	pooled	F55, F56, F83
	Ophiura ophiura	Serpent brittle star	June, September	F55, F56, F83
	Sargassum muticum	Strangleweed	pooled	F9
Acinetobacter baumannii	*Alcyonium digitatum*	Dead man's finger	September	F4, F5
	Echiichthys vipera	Lesser weever	pooled	
Pseudomonas aeruginosa	*Alcyonium digitatum*	Dead man's finger	September	F5
Candida albicans	*Alcyonium digitatum*	Dead man's finger	September	F3
	Crangon crangon	Common shrimp	December	F83
	Cyanea lamarckii	Blue jellyfish	June	F2
	Liocarcinus depurator	Harbour crab	pooled	F80

4.6.3. Biofilm inhibition and eradication

The fractions that showed highest activity against planktonic bacteria were also tested for their ability to inhibit biofilm formation in 96-well plates. Fractions that resulted in at least 1 log reduction compared to an untreated control are shown in Table 8 with the fractions showing the highest biofilm inhibition on top.

Table 8: Best-performing fractions in terms of biofilm inhibition (i.e. fractions giving at least 1 log reduction compared to the untreated control) in order of performance.

Planktonic microorganism	Species	Common name	Month	Fraction	Log reduction
Staphylococcus aureus	*Echiichthys vipera*	Lesser weever	June	F2	9.84
	Ophiura ophiura	Serpent brittle star	June	F55	5.76
	Ophiura albida	Serpent's table brittle star	pooled	F55	5.55
	Ophiura ophiura	Serpent brittle star	June	F56	4.99
	Crangon crangon	Common shrimp	December	F83	4.76
	Alcyonium digitatum	Dead man's finger	September	F5	4.60
	Ophiura albida	Serpent's table brittle star	pooled	F56	4.42
	Ophiura albida	Serpent's table brittle star	pooled	F83	3.12
	Alcyonium digitatum	Dead man's finger	September	F4	2.80
	Crangon crangon	Common shrimp	December	F84	2.45
	Ophiura ophiura	Serpent brittle star	June	F83	2.29
	Ophiura ophiura	Serpent brittle star	September	F83	1.91
	Crangon crangon	Common shrimp	December	F85	1.78
	Asterias rubens	Common starfish	June	F83	1.71
	Ophiura ophiura	Serpent brittle star	September	F46	1.60

Crangon crangon	Common shrimp	December	F86	1.53
Crangon crangon	Common shrimp	December	F87	1.22
Asterias rubens	Common starfish	September	F84	1.21
Ophiura ophiura	Serpent brittle star	September	F47	1.12
Ophiura albida	Serpent's table brittle star	pooled	F41	1.04

Biofilm eradication was also assessed, and fractions that resulted in at least 1 log reduction compared to an untreated control are shown in Table 9.

Table 9: Best-performing fractions in terms of biofilm eradication (i.e. fractions giving at least 1 log reduction compared to the untreated control).

Planktonic microorganism	Species	Common name	Month	Fraction	Log reduction
Staphylococcus aureus	*Ophiura ophiura*	Serpent brittle star	June	F83	4.05
	Ophiura ophiura	Serpent brittle star	June	F56	3.84
	Ophiura albida	Serpent's table brittle star	pooled	F83	3.24
	Ophiura ophiura	Serpent brittle star	June	F56	4.99
	Ophiura ophiura	Serpent brittle star	September	F83	2.81
	Ophiura albida	Serpent's table brittle star	pooled	F55	1.04
	Ophiura albida	Serpent's table brittle star	pooled	F56	1.46
	Ophiura ophiura	Serpent brittle star	June	F55	1.42
	Crangon crangon	Common shrimp	December	F86	1.26
	Crangon crangon	Common shrimp	December	F83	1.22

4.6.4. Growth inhibitory activity and killing activity towards planktonic cells of subfractions

Based on the above results, subfractions were tested from 8 fractions from 5 organisms (selection from all subfractions prepared as presented in Table 2). The growth inhibitory and bactericidal activity is summarized in Table 10 and 11. For several fractions that showed activity in the initial screening, antimicrobial activity could not be assigned to any of the subfractions. The reason for this is at present unclear. Due to limited resources, not all of the promising fractions presented in Table 6 and 7 could be sub-fractionated and further tested for growth inhibitory and killing activity towards planktonic cells.

Table 10: Growth inhibition of planktonic microorganisms by selected subfractions. All results of the activity against at least one of the test organisms is shown, including the absence of any activity (cells in grey).

Planktonic microorganism	Species	Common name	Month	Fraction	Sub-fraction	% inhibition of growth when tested in a ½ dilution	Fraction MIC (fold dilution of original sample)	Subfraction MIC
Staphylococcus aureus	Alcyonium digitatum	Dead man's finger	September	F4	SF5	49%	1/4	>1/2
	Alcyonium digitatum	Dead man's finger	September	F5	SF5	100%	1/16	1/8
	Echiichthys vipera	*Lesser weever*	June	F2	SF4	99%	1/8	-*

	Ophiura albida	Serpent's table brittle star	June	F55	no hits	-	1/16	-
	Ophiura albida	Serpent's table brittle star	June	F83	no hits	-	1/64	-
	Ophiura ophiura	Serpent brittle star	June	F55	no hits	-	1/32	-
	Ophiura ophiura	Serpent brittle star	June	F83	no hits	-	1/19	-
Acinetobacter baumannii	*Echiichthys vipera*	*Lesser weever*	June	F2	SF3	100%	1/16	1/2
	Echiichthys vipera	*Lesser weever*	June	F2	SF4	100%	1/16	1/8
Pseudomonas aeruginosa	Alcyonium digitatum	Dead man's finger	September	F4	SF5	98%	1/16	1/2
	Alcyonium digitatum	Dead man's finger	September	F5	SF5	100%	1/16	1/8
Candida albicans	Lanice conchilega	sand mason worm	all months	F80	no hits	-	1/4	-

Table 11: Bactericidal activity of subfractions. -: no growth observed at this concentration, suggesting killing activity; +: growth observed at this concentration, suggesting no killing activity.

							Incubation time	
Planktonic microorganism	**Species**	**Common name**	**Month**	**Fraction**	**Sub-fraction**	**Concentration**	**+24h**	**+48h**
Staphylococcus aureus	*Alcyonium digitatum*	Dead man's finger	September	F4	SF5	1/2	+	+
	Alcyonium digitatum	Dead man's finger	September	F5	SF5	1/2	+	+
	Alcyonium digitatum	Dead man's finger	September	F5	SF5	1/4	+	+
Acinetobacter baumannii	*Echiichthys vipera*	Lesser weever	June	F2	SF3	1/2	+	+
	Echiichthys vipera	Lesser weever	June	F2	SF4	1/2	-	-
	Echiichthys vipera	Lesser weever	June	F2	SF4	1/4	-	-
	Echiichthys vipera	Lesser weever	June	F2	SF4	1/8	+	+
Pseudomonas aeruginosa	*Alcyonium digitatum*	Dead man's finger	September	F4	SF5	1/2	+	+
	Alcyonium digitatum	Dead man's finger	September	F5	SF5	1/2	+	+
	Alcyonium digitatum	Dead man's finger	September	F5	SF5	1/4	+	+
	Alcyonium digitatum	Dead man's finger	September	F5	SF5	1/8	+	+

4.6.5. Conclusions from antimicrobial assays

Antimicrobial activity against *Candida albicans*, *Staphylococcus aureus*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* (for some cases) was carried out for 31 species. Antimicrobial tests included the determination of minimal inhibitory concentration and minimal bactericidal/fungicidal concentrations. In the second research phase, the antibiofilm activity of selected compounds was evaluated. Antimicrobial activity is found in a wide range of organisms and fractions but is predominantly found in early (F2-F4) and late (F80-F85) fractions. This pattern occurred in a wide taxonomic distribution suggesting that antimicrobial activity in these 'early' and 'late' fractions is aspecific, and possibly attributable to high salt concentration ('early' fractions) and the presence of surfactants ('late' fractions). Follow-up research should be carried out to confirm this.

Based on the results, 8 positive hits from 5 species were identified for further subfractionation purposes:

- *Alcyonium digitatum* - Sept (F4 & F5)
- *Echiichtys vipera* - June (F2)
- *Ophiura albida* (F55 & F83)
- *Ophiura ophiura* - June (F55 & F83)
- *Liocarcinus depurator* (F80)

Several subfractions revealed a positive hit again, showing that subfractionation of positive fractions is possible without losing the bioactive compound, and resulting in a much less complex subfractions containing only a few compounds of which one of them is the bioactive compound. Some results also show that the antimicrobial activity of positive fractions was lost during subfractionation. The reason for this is at present unclear, but could be related to unstable compounds (degradation) or too low concentrations after subfractionation. Due to limited resources, not all of the promising fractions could be sub-fractionated and further testing for growth inhibitory and killing activity towards planktonic cells should be continued in follow-up research, especially the hit fractions outside the ‘early’ and ‘late’ regions, which are:

- *Asterias rubens* (F48, F50 & F58)
- *Echinocardium cordatum* (F45, F46 & F51)
- *Ophiura albida* (F38 & F59)
- *Ophiura ophiura* (F38 – F64)
- *Sargassum muticum* (F8 – F20)
- *Lanice conchilega* (F16 – F17)
- *Mytilus edulis* (F15)

5. Valorisation

The hit species found from the bioactivity screening were cross-linked with the bioprospection index cards in order to identify the most likely application(s) for the detected active fractions. For some species, evidence of bioactivity was already reported in literature, while for others the discovery is quite novel. Moreover, whenever cultivation opportunities were known for a certain species, the corresponding publication was included in the bioprospection index cards. For five species (*Asterias rubens*, *Echinocardium cordatum, Ophiura ophiura, Sargassum muticum* and *Ophiura albida*) and five applications (cosmetics, pharmaceuticals, antifouling, functional ingredient and hygienic packaging), a use case was developed. The species were chosen based on the most promising results from the antimicrobial and electrophysiological assays (Deliverable 5.2-5.3). This resulted in **a set of five use cases for promising hit species** that were described in a fact sheet.

From an application perspective, the Strategic Advisory Board identified the use case "antifouling solutions in offshore and onshore aquaculture facilities" as most promising. In a brainstorm session the assumptions that need to be validated regarding problem fit, solution fit and business model fit were identified. This resulted in a concept note for a feasibility study. However, it became clear that there is still a large gap between the research results and the industrial applications. **Therefore, a next step needs to be taken by the research partners in identifying and purifying the compounds that were found in the "hit" fractions and sub-fractions. Fractionation of the active mixtures to generate subfractions with a less complex metabolic composition is highly desired in order to perform a more targeted study on the biotechnological potential of the compounds produced by a certain organism. This knowledge is important for the industry in order to be able to register new products and to develop new processing technologies/partnerships.**

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7. Supplementary tables

S-table 1: Summary of the results obtained during screening against planktonic microorganisms. Only marine organisms and fractions that showed a hit against at least one of the test organisms is shown. +: fraction with moderate activity; ++: fraction with high activity, i.e. MIC is lower than or equal to 1/8 dilution.

Organism	Batch	Fraction	*S. aureus*	*A. baumannii*	*P. aeruginosa*	*C. albicans*
Aurelia Aurita (Common jellyfish)	AAU_02	F2	+	+	+	+
		F3	+	+	+	+
		F4	+	+	+	+
Alcyonium digitatum (Dead man's finger)	AD_SEPT_02	F2	+	+	++	+
		F3	+	+	++	+
		F4	+	++	++	
		F5	+	++	++	
Alloteuthis subulata (European common squid)	AS_SEPT_02	F2		+	+	
		F3		+		
Asterias rubens (Common starfish)	PROBIO_AR_2	F83	+			
	AR_JUNE_02	F2		+		
		F3		+	+	
		F59	+			
		F83	++			
		F84	+			
	AR_SEPT_02	F2		+	+	
		F3		+	+	
		F4		+		
		F48	+			
		F50	+			
		F59	+			
		F80	+			
		F83	+	+		+
		F84	+			
		F85	+			
Botrylloides violaceus (Colonial sea squirt)	BV_02	F2	+	+	+	+
		F3	+	+	+	+
		F4		+	+	
Chrysaora hyoscella (Compass jellyfish)	CH_JUNE_02	F2		+	+	+
		F3	+	+	+	+
		F4	+	+	+	+
		F84	+			
	CH_SEPT_02	F2		+	+	
		F3		+	+	+
		F4		+	+	
Codium Fragile (Sponge seaweed)	CFr_02	F2		+	+	+
		F3		+	+	
		F4		+	+	
Crangon crangon (Common shrimp)	CC_SEPT_02	F2		+	+	
		F3		+	+	
		F80	+			
		F83	+			
		F84	+			
		F85	+			
	CC_DEC_02	F2		+	+	
		F3		+	+	
		F4		+		
		F83	++	+	+	+

		F84	++	+		+
		F85	++	+		
		F86	++			
		F87	+			
		F88	+			
Crepidula fornicata (Common slipper shell)	CF_02	F2		+		
		F3		+		
		F83	+			
		F85	+			
		F88	+			
Cyanea lamarckii (Blue jellyfish)	CL_JUNE_02	F2	+	+	+	+
		F3	+	+	+	+
		F4		+	+	
		F83	+			
		F84	+			
Ensis spp. (Razor clam spp.)	ENS_02					
Echiichthys vipera (Lesser weever)	EV_JUNE_02	F2	++	++	++	+
		F3	+	+	+	
		F4	+	+	+	
		F84	+			
	EV_SEPT_02	F2		+	+	
		F3		+	+	
		F4		+	+	
		F6				+
Echinocardium cordatum (Sea potato)	EC_JUNE_02	F2		+		
		F45	+			
		F46	+			
		F81	+			
		F82	+			
	EC_SEPT_02	F2		+	+	
		F3		+	+	+
		F4		+	+	
		F5				+
		F51	+			
Ectopleura larynx (Ringed tubularian)	PROBIO_EA_2	F3		+		
		F82	+			
		F83	+			
		F84	+			
		F85	++			
	ELa_JUNE_02	F2		+	+	
		F3		+	+	
		F4		+	+	
		F5		+	+	
Halichondria panicea (Breadcrumb sponge)	HP_02	F2	+	+	+	+
		F3	+	+	+	+
		F4	+	+	+	+
Haliclona oculata (Mermaid's glove)	HO_02	F2		+	+	+
		F3		+	+	
		F4		+	+	
Lanice conchilega (Sand mason worm)	LC_02	F3	+	+	+	+
		F4	+	+	+	+
		F5	+	+	+	+
		F6		+		
		F16		+	+	
		F17		+		
	LD_02	F80				++

Liocarcinus depurator (Harbour crab)		F83	++			
		F84	++			
		F85	+			
Liocarcinus holsatus (Flying crab)	LH_JUNE_02	F2		+		
		F3		+		
		F83	+			
		F84	++	+		
		F85	+			
	LH_SEPT_02	F83	+			
		F84	+			
Metridium senile (Frilled anemone)	MS_JUNSEPT_02	F2		+	+	
		F3		+	+	
		F4		+		
Mytilus edulis (Blue mussel)	ME_02	F3	+	+	+	+
		F4	+	+	+	+
		F5		+	+	
		F6	+	+		
	PROBIO_ME_2	F2				+
		F3				+
		F5				+
		F6				+
		F15				+
Ophiura albida (Serpent's table brittle star)	OA_02	F38	+			
		F39	+			
		F40	+			
		F41	+			
		F42	+			
		F43	+			
		F44	+			
		F45	+			
		F55	++			
		F56	++			
		F57	+			
		F58	+			
		F59	+			
		F83	++	+		
		F84	+	+		
		F85	+	+		
Ophiura ophiura (Serpent brittle star)	OO_JUNE_02	F38	+			
		F39	+			
		F40	+			
		F41	+			
		F42	+			
		F43	+			
		F44	+			
		F45	+			
		F46	+			
		F49	+			
		F50	+			
		F51	+			
		F52	+			
		F53	+			
		F54	+			
		F55	++			
		F56	++			
		F57	+			
		F58	+			

		F59	+			
		F60	+			
		F61	+			
		F62	+			
		F63	+			
		F64	+			
		F83	++			
	OO_SEPT_02	F46	+			
		F47	+			
		F83	++			
Pleurobrachia pileus (Sea gooseberry)	Ppi_02	F2	+	+	+	+
		F3	+	+	+	+
		F4		+	+	+
Psammechinus miliaris (Green sea urchin)	PM_JUNE_02	F2		+		
		F3		+		
Sagartia spp. (Cave-dwelling anemon)	Sgs_02	F2		+	+	
		F3		+	+	
Sargassum muticum (Strangleweed)	SM_02	F2	+	+	+	+
		F3	+	+	+	+
		F4		+	+	
		F6	+			
		F7	++			
		F8	+			
		F9	++			
		F10	+			
		F11	+			
		F12	+			
		F13	+			
		F14	+			
		F15	+			
		F16	+			
		F17	+			
		F18	+			
		F19	+			
		F20	+			
Styela clava (clubbed tunicate)	SCV_02	F2	+	+	+	+
		F3		+	+	+
		F4	+	+	+	+
Tricellaria inopinata	TrIn_02	F2		+	+	
		F3	+	+	+	+
		F4	+	+	+	+
		F5		+		
Tritia reticulata (Netted dog whelk)	TR_02	F2		+	+	
		F3		+	+	+
Ulva pseudocurvata	UP_02	F2	+	+	+	+
		F3	+	+	+	+
		F4	+	+	+	+
		F5		+	+	+

S-table 2: Bactericidal activity of selected fractions against S. aureus. -: no growth observed at this concentration, suggesting killling activity; +: growth observed at this concentration, suggesting no killing activity.

Organism	Batch	Fraction	Test concentration (expressed as fold MIC)	Incubation time	
				+24h	+48h
Asterias rubens (Common starfish)	AR_JUNE_02	F83	1/4 1/8	- +	-
Crangon crangon (Common shrimp)	CC_DEC_02	F83	1/16 1/32	- -	- +
		F84	1/16	-	-
		F85	1/4 1/8	- +	+
		F86	1/4 1/8 1/16	- - +	- +
Echiichthys vipera (Lesser weever)	EV_JUNE_02	F2	1/4 1/8	- +	-
Ophiura albida (Serpent's table brittle star)	OA_02	F55	1/8 1/16	- +	-
		F56	1/4 1/8	- +	-
		F83	1/64	-	-
Ophiura ophiura (Serpent brittle star)	OO_JUNE_02	F55	1/16 1/32	- +	-
		F56	1/8	-	-
		F83	1/4 1/8 1/16	- - +	- +
	OO_SEPT_02	F83	1/2 1/4 1/8	- - +	- +
Sargassum muticum (Strangleweed)	SM_02	F7	1/2 1/4 1/8	+ + +	
		F9	1/2 1/4 1/8	- + +	

S-table 3: Bactericidal activity of selected fractions against A. baumannii and P. aeruginosa. -: no growth observed at this concentration, suggesting killling activity; +: growth observed at this concentration, suggesting no killing activity.

Organism	Batch	Fraction	Species	Test concentration (expressed as fold MIC)	Incubation time	
					+24h	+48h
Alcyonium digitatum (Dead man's finger)	AD_SEPT_02	F3	*P. aeruginosa*	1/2 1/4 1/8	+ + +	
		F4	*A. baumannii*	1/4 1/8	- +	-
			P. aeruginosa	1/2 1/4 1/8 1/16	+ + + +	
		F5	*A. baumannii*	1/8 1/16	- +	-
			P. aeruginosa	1/4 1/8 1/16	- + +	+
Echiichthys vipera (Lesser weever)	EV_JUNE_02	F2	*A. baumannii*	1/8 1/16	- +	-
			P. aeruginosa	1/8	-	-
Ophiura albida (Serpent's table brittle star)	OA_02	F83	*A. baumannii*	1/2 1/4	+ +	

S-table 4: Fungicidal activity of selected fractions against C. albicans. -: no growth observed at this concentration, suggesting killling activity; +: growth observed at this concentration, suggesting no killing activity.

Organism	Batch	Fraction	Test concentration (expressed as fold MIC)	Incubation time	
				+24h	+48h
Aurelia Aurita (Common jellyfish)	AAU_02	**F3**	1/2	+	
Alcyonium digitatum (Dead man's finger)	AD_SEPT_02	**F3**	1/2	-	-
Crangon crangon (Common shrimp)	CC_DEC_02	**F83**	1/2	-	-
Cyanea lamarckii (Blue jellyfish)	CL_JUNE_02	**F2**	1/2	-	-
		F3	1/2	+	
Lanice conchilega (Sand mason worm)	LC_02	**F2**	1/2	+	
		F3	1/2	+	
Liocarcinus depurator (Harbour crab)	LD_02	**F80**	1/2 1/4	- -	- -
Halichondria panicea (Breadcrumb sponge)	HP_02	**F2**	1/2	+	
		F3	1/2	+	
Sargassum muticum (Strangleweed)	SM_02	**F2**	1/2	+	
		F3	1/2	+	
Ulva pseudocurvata	UP_02	**F2**	1/2	+	
		F3	1/2	+	
Tricellaria inopinata	Trln_02	**F3**	1/2	+	
		F4	1/2	+	