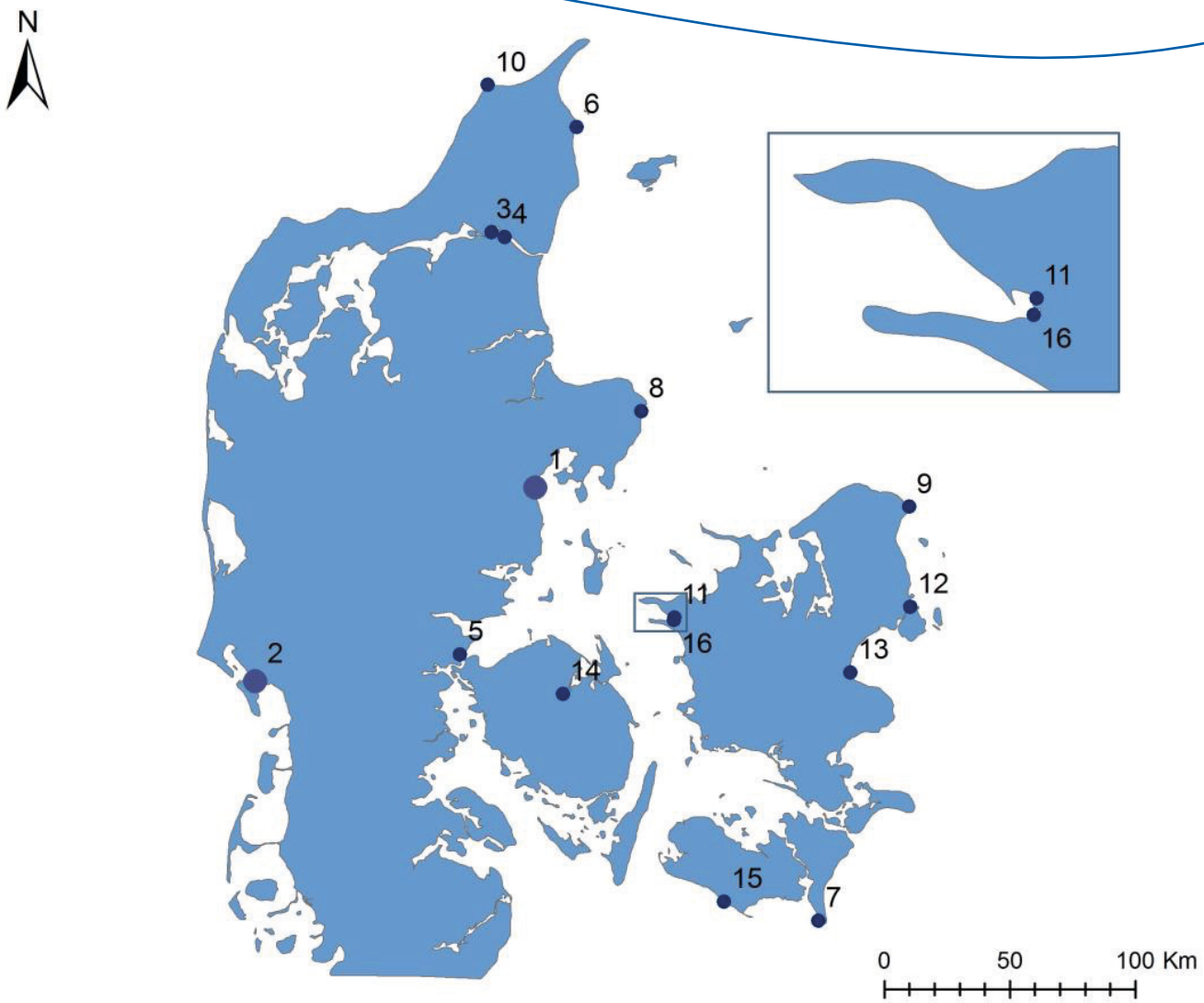
RAPPORT L.NR. 7175-2017

Sampling protocol for monitoring of non-indigenous species in selected Danish harbours



Den Europæiske Union og Den Europæiske Hav- og Fiskerifond har via Landbrugs- og Fiskeristyrelsen finansieret nærværende projekt "Monitoring of non-indigenous species in selected Danish harbours" (MONIS 4) med MST som faglig kontakt.

NIVA Denmark Water Research

REPORT

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Summary

The sampling protocol is a product of the MONIS 4 project, or in full "Monitoring of non-indigenous species in selected Danish harbours". Sampling will take place using a broad range of methods, both conventional sampling methods and sampling of water and subsequent analyses of DNA remains of a total of 20 species on the Danish non-indigenous target species list. Groups of organisms covered by this study include: (1) phytoplankton, (2) zooplankton, (3) softbottom communities (infauna and epifauna), (4) hardbottom communities (both flora and fauna), as well as (5) fish. The results of this comprehensive sampling effort in 16 Danish harbours is planned to be reported by Summer 2018. Data will after publication of the report be submitted to relevant data repositories.

Four keywords	Fire emneord
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Preface

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Groups of organisms covered by the study include: (1) phytoplankton, (2) zooplankton, (3) softbottom communities (infauna and epifauna), (4) hardbottom communities (both flora and fauna), as well as (5) fish. Sampling will take place during summer of 2017.

The results of this comprehensive sampling effort in 16 Danish harbours is planned to be reported by Summer 2018. Data will after publication of the report be submitted to relevant data repositories.

Copenhagen, 11 July 2017

Jesper H. Andersen

1 Background and objectives

NIVA Denmark has in December 2016 and in collaboration with a consortium including Amphi Consult Aps, the Danish Natural History Museum (NHM), DTU Aqua, LITEHAUS Aps and the Norwegian Institute for Water Research (NIVA) won a tender on monitoring of non-indigenous species (NIS) in selected Danish harbours.

The partnership has worked together on these and similar issues since 2014, mostly with the following suite of projects, all NIS related:

- MONIS 1, or in full 'Monitoring of non-indigenous species in Danish marine waters. Background and proposals for a monitoring strategy and a monitoring network'. For details, see Andersen et al. (2014).
- MONIS 2, or in full 'Steps toward nation-wide monitoring of non-indigenous species in Danish marine waters under the Marine Strategy Framework Directive'. For details, see Andersen et al. (2016).
- MONIS 3, or in full 'Monitoring of non-indigenous species in Danish marine waters: Development of species-specific operational test systems'. This project is ongoing and the result will be published in December 2017.

MONIS 4 has an overarching goal of monitoring 16 selected Danish harbour with respect to the occurrence of non-indigenous species and thus establish a baseline. MONIS 4 is organised as outlined in Figure 1.

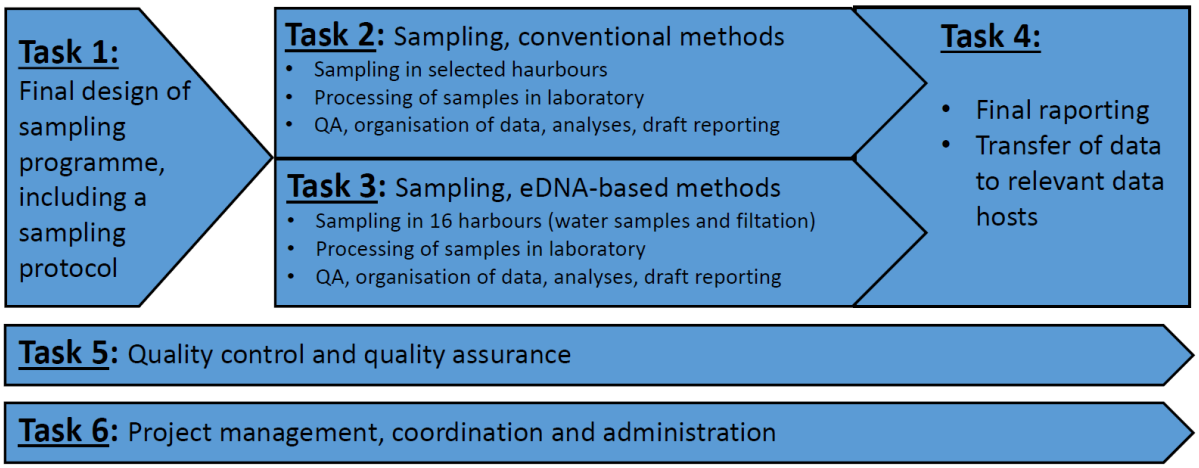

Figure 1: *Organisation and workflow of MONIS 4.*

The monitoring in the context of MONIS 4 will take place in the following harbours (Figure 2):

1. Aarhus harbour
2. Esbjerg harbour
3. Aalborg Portland harbour
4. Aalborg harbour
5. Fredericia harbour
6. Frederikshavn harbour
7. Gedser harbour
8. Grenå harbour
9. Helsingør harbour

10. Hirtshals harbour
11. Kalundborg harbour
12. Copenhagen harbour
13. Køge harbour
14. Odense harbour
15. Rødby Ferry harbour
16. Statoil harbour (Kalundborg

The above harbours have been selected based on the outcomes and recommendation of the MONIS 1 project (see Andersen et al. 2015), where 17 harbours were identified as 'priority harbours'. Rønne harbour on the island of Bornholm, has been omitted for practical reasons.

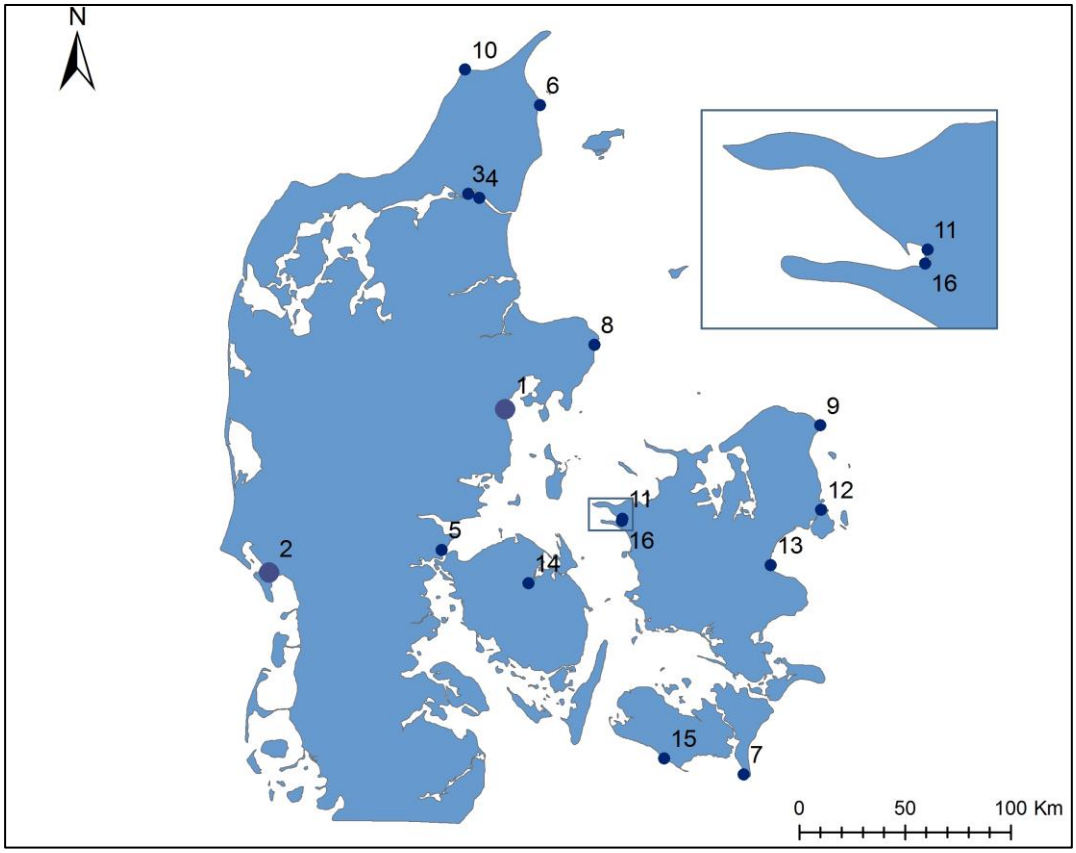

Figure 2: *Map of the harbours included in the MONIS 4 study.*

The objectives of this sampling protocol are:

- to outline the sampling methods and the equipment to be applied,
- to determine the sampling stations in each of the selected 16 Danish harbours, and
- to suggest and agree on exact dates for sampling in each of the 16 selected harbours.

We believe that we have established not only a joint and well-coordinated sampling programme but also developed a platform for a successful execution of MONIS 4 and future monitoring activities following up on this baseline study.

2 Sampling methodology

In the MONIS 4 project, sampling will include both sampling using conventional in situ methods (section 2.1) and biomolecular methods (section 2.2).

2.1 Conventional sampling methods

Sampling will be carried out with respect to the following groups of organisms:

- Plankton (phyto- and zooplankton)
- Softbottom communities (sediment infauna and epifauna)
- Hardbottom communities (fouling organisms)
- Fish

2.1.1 Phyto- and zooplankton

Sampling of phyto- and zooplankton will take place in Aarhus Harbour and Esbjerg Harbour, the two largest industrial harbours in Denmark. In each of the harbours, sampling will be carried out in accordance with the Combine Manual for Marine Monitoring, Annex 6 and the Joint Harmonised Procedure of OSPAR[1], at 3 sampling stations placed in different sections of the harbour two times, once in early summer (June) and once in late summer (August/September).

Phytoplankton
Sampling may be carried out from a pier or boat.

Equipment; water sampler (Figure 3), bottle (4 L for mixing the discrete water samples), plankton net (10 mesh) (Figure 4) fitted with a ca 500 g weight, 100 ml bottles for preserved water and net samples, Lugol's solution, white Secchi disk (Figure 5).

Figure 3: *Water sampler.*

Figure 4: *Plankton net.*

Figure 5: *Secchi disc.*

[1] With the exception from the Combine protocol that zooplankton is preserved in ethanol instead of formaldehyde.

Each station; Temperature, salinity and oxygen is measured using a SAIV CTD (SD204, see http://www.saivas.no/visartikkel.asp?art=2).

To estimate the extent of the euphotic zone, a white Secchi disk is released into the water and lowered until it is no longer visible (or in very shallow waters until it touches the bottom) and the depth is recorded.

A plankton net (10 µm mesh) is lowered to the depth corresponding to that recorded by the Secchi disk measurement and carefully hauled (< 0.3 m/sec) to the surface. To collect all organisms into the sample bottle at the bottom of the net, the net will be rinsed by raising and lowering it several times in the surface water while making sure that the upper ring is kept above the surface (multiple hauls may have to be carried out to collect sufficient material).

Water will be collected with a water sampler from 4 depths; the surface, 2.5 m, 7 m and 10 m (in shallow areas the deeper depths must be omitted) and gently mixed in a bottle. A subsample of 100 ml will be preserved in Lugol's solution (1 % final concentration). Remember to label the samples properly (harbour, station, date).

Cleaning of the net: After sampling the plankton net should be soaked for ca 20 minutes in warm tap water, rinsed in tap water and dried.

Zooplankton

Sampling may be done from a pier. However, sampling is best done from a boat, since it may be difficult to avoid scraping vertical surfaces if done from a pier or wharf. The time for sampling should be close to high tide.

Equipment: WP2 200 µm net, WP2 500 µm net (figure 4), (flow meter), weight to keep the net vertically, 250-500 ml sample bottles, labels, squirt bottle, funnel, ethanol 96% (not denatured), glass tank/small aquarium, Virkon S for disinfection, tub for soaking nets and line during disinfection

Each station: Two samples should be taken at each station - separate samples with fine (200 µm) and coarse (500 µm) nets. Vertical hauls from near bottom to surface. Lower the net to desired depth (do not touch bottom!), and haul slowly towards the surface at ca. 0.5 m/s for both net types. Several hauls may be combined in one sample. This is important if the hauls are short and few animals are caught in each haul.

Jelly plankton must be identified alive. Pour sample gently into a glass jar/aquarium for observation (add sea water as necessary). An important target species is the American ctenophore *Mnemiopsis leydyi*, which can easily be confused with the native *Bolinopsis infundibulum*. If the species can not be identified, a digital photo should be taken.

Afterwards, remove jellies as best you can, and filter the samples again (in the plankton net or a filter with the same mesh size). If the plankton net is used for filtration, remove as much water as possible. Use a squirt bottle with ethanol and a funnel to transfer the animals into the sample bottle. Fill up with ethanol. Label the samples properly (harbour, station, date, net mesh size).

Disinfection: After sampling, plankton nets, weight, flow meter and line must be disinfected by soaking 20 minutes in a Virkon S solution, or some alternative method (chlorine or 70 % ethanol). Rinse in tap water and leave to dry.

Combine manual for Marine Monitoring in the COMBINE Programme of HELCOM. Annex 6.

Joint Harmonised Procedure for the Contracting Parties of OSPAR and HELCOM on the granting of exemptions under International Convention for the Control and Management of Ships' Ballast Water and

Sediments, Regulation A-4. Adopted as OSPAR Agreement 2013-09 and by HELCOM Ministerial Meeting Copenhagen 3 October 2013.Amended by HELCOM 2015 (March) and OSPAR Agreement 2015-XX.

2.1.2 Softbottom communities

Sampling of softbottom species communities will be performed according to the HELCOM-OSPAR JHP-procedure (HELCOM-OSPAR 2015). In each harbour, three sampling stations ('sites' according to the JHP procedure description) will be allocated in different parts of the harbour. The sampling will be performed in late summer (September).

Sediment infauna
A minimum of three grab samples will be taken at each sampling station. The samples will be located at least 15 m distance from each other and at varying distances from docks and piers. The samples will be taken from a small craft using a hand-operated 0.025 m² Ekman (Figure 6) or van Veen (Figure 7) grab.

Figure 6: *Ekman grab: 15x15 cm=0.025 m²*

Figure 7: V*an Veen grab: 14x20 cm = 0,028 m²*

Choice of gear will depend on the sediment structure and penetration, but a minimum of 1/3 grab filling will be required for a sample to be approved. In addition, supplementary samples will be taken using a Kajak corer (Figure 8) to ensure sufficiently deep penetration in the sediment.

Figure 8: *Kajak corer: 5.5 cm in diameter.*

Each sample will consist of three or four grab hauls that are mixed to cover a sampling area of minimum 0.1 m². The samples are sieved on 0.5 mm screens and fixed in 4-6 % formaldehyde solution in seawater. A visual description of the sediment catch (colour, smell, larger objects) will be performed before sieving. At each sampling station, one or two supplementary samples for ethanol-fixation (96%) will be collected in case of later molecular genetic analyses of selected species. In addition, a sediment sample for analysis of grain size and organic content (total organic carbon, total nitrogen) will be taken and kept for analysis. Analysis will be carried out if such analyses are relevant.

Samples will be sorted and identified at NIVAs laboratory in Oslo. All species will be identified to the lowest possible taxonomic level. The analyses will be carried out in accordance with NIVAs accredited routines for processing of quantitative soft bottom samples.

Sediment epifauna
A minimum of three samples of sediment epifauna (sessile and slow-moving organisms) will be collected simultaneously with the grab samples at each sampling station in the harbours. The samples will be taken with a 20 cm wide light-weight hand operated dredge (Figure 9) from a small craft.

Figure 9: *Dredge: 40 x 20 cm.*

The dredge will be towed 20-30 m along the bottom. The sediment catch will be sieved on 0.5 mm screens and fixed in formaldehyde-solution and/or ethanol.

Samples will be sorted and identified at NIVAs laboratory in Oslo. Sorting and identification will follow the same routines as the quantitative grab samples.

Mobile epifauna
Mobile epifauna (crabs and prawns) will be collected using traps. Two types of traps will be used, one with a rather coarse mesh netting (crab trap, 1-2 cm mesh size) and one with a fine mesh (minnow trap, ca 5 mm mesh size). Three traps of both trap type will be deployed at each sampling station. The traps will be baited and deployed for two days (48 h).

After trap retrieval, the catch of crabs, fish and other mobile fauna will be identified as far as possible in the field (common native species), measured and weighed (crabs: carapax width; prawns: carapax length). Other material will be taken to a laboratory for identification, measured and weighed. Preferentially the material will be brought fresh to the laboratory, but in case of long transport, the material will be frozen or preserved in formalin solution.

2.1.3 Hardbottom communities

Sampling of hardbottom species communities will be performed according to the HELCOM-OSPAR JHP-procedure (HELCOM-OSPAR 2015). In each harbour, sampling will take place at three locations in the vicinity of the sampling stations for softbottom communities.

Fouling organisms – scraping of hard substrates
Scraped samples will be collected from at least nine different sites in each harbor (three in each of three survey locations). Each sample will cover approximately 0.1 m^2 surface area and the samples will be removed from the substrates from close to the water surface to depths of <1 meter. The geographical distance between each sample should be >10 meters. The samples will be collected using a scraper with a pocket-net attached to a pole which will be operated from a boat or from the dock. Sampled material will be transferred from the net to a white plastic tray filled with sea water and examined immediately. The sampling will be performed in late summer (September).

Each scraped sample will be photographed. The abundance of the species will be estimated according to a semi-quantitative scale: 1 - " individual finding" 2 - "spread distribution" 3 - "normal distribution" and 4 - "dominant distribution". Species that cannot be identified in the field or are not previously recorded in Denmark will be removed from the sample and brought to the laboratory to be examined microscopically. Species that cannot be identified immediately or individuals to be saved for documentation will be preserved in 90% ethanol.

In addition, selected substrates (floating bridges, fender constructions, ropes etc.) will be examined for alien species using a 'rapid assessment survey' (RAS) technique. RAS is a time efficient method where submerged structures in marinas, such as pontoons, floats and pier elements, are examined for the presence of species that may be expected to occur ('target species'). ID – sheets with picture and description of relevant target species will be brought in the field. The method is described in Minchin et. al. (2007) and HELCOM-OSPAR (2015). To examine substrates in deeper water, a drop-camera with video-recording will be used, provided the water is not too turbid.

Settling plates
Fouling plates in PVC will be used to examine colonization of alien species on artificial substrates. Fouling plates will be deployed at nine sites (three in each of three survey locations) in each harbour. The plate units will be constructed of three plates measuring 14 cm x 14 cm fixed on a polypropylene rope (Figure 10). The settlement plates will be sanded lightly with a sandpaper on both sides to create a suitable surface for colonizing organisms. A hole will be drilled through the middle of the plates so that a polypropylene rope can be threaded through. The plates position on the rope will be secured with a knot above and below each of the plates and the length of the rope will be adjusted so that the three plates achieve a depth of 1, 3 and 7 meters when the unit are deployed in the water. At sites where the water depth is less than 8 meters at low tide, the deepest plate will be removed and the length of rope will be adjusted accordingly. At the end of the rope a weight will be attached to weigh down the rig and ensure that the rope remains tight and achieves an approximately vertical position in the water column. The weight will be fixed approximately 1 m from the deepest settlement plate.

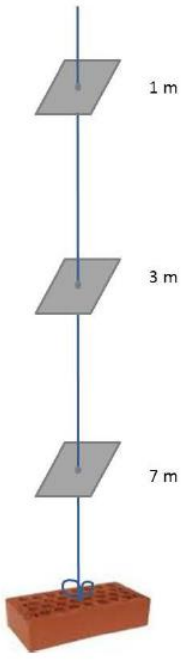

Figure 10: *Settlement plates.*

The plate units should be deployed at sites where they are not readily available or visible to people or in danger of being damaged by boat traffic. Preferably the ropes will be attached underneath floating docks to prevent settlement plates from drying at low tide. Tidal differences should be accounted for when the rigs are deployed to prevent the rope from touching the seabed during low tide. Plate units will be marked with waterproof labels and GPS position will be noted at sites where the rigs are deployed.

Colonization of settlement plates follows four phases (Wahl 1989):

1. Coating of organic film from substances in sea forms immediately
2. Primary bacterial formation
3. Secondary colonization of unicellular eukaryotes (diatoms, protozoa, fungi)
4. Tertiary colonization of multicellular eukaryotes (macroalgae, barnacles, mussels etc.)

The tertiary colonization starts after approximately one week. The greatest abundance of pelagic larvae usually occurs in the spring followed by settlement throughout spring and summer. Large brown algae form spores that settles during winter, while several of the one-year algae recruits in the spring and early summer.

The settlement plates will be deployed in June and retrieved in mid-September. Plates will be carefully detached from the rope and photographed before they are placed in separate, pre-labeled zip-lock bags. Sufficient seawater will be added so the material keeps humid. Plates will be kept in a cooler during transport to the laboratory. Species that cannot be identified immediately or individuals to be saved for documentation will be preserved in 90% ethanol. If target species are observed on the settlement plates their coverage will be estimated using the following semi-quantitative scale: 1 = 'individual finding', 2 = 'spread distribution', 3 = 'normal distribution' and 4 = 'dominant distribution'.

2.1.4 Fish, standard methods

Fish will be sampled using gillnets and fyke-nets (Figure 11).

Multi-meshed gillnets: Seven different panels will be used to make up a gillnet series. The panels have the following mesh sizes: 11,0 14.3, 18.6, 24.2, 31.4, 53.1 and 89.7 mm each measuring 1.5 m in height. The lengths of each panel are 2.95, 5.30, 5.70, 5.70, 6.00, 11.60 and 11.60 m. Each panel is separated by about 1 m resulting in gillnets of around 55 m in length. If there is not sufficient space the gillnet will be divided into smaller sections.

Fyke-nets have a mesh size of 18 mm and are 42 cm in height and with a 6.5 m leader. One fyke-net consists of the leader and two cod-ends.

In each harbour, one gill-net and one fyke-net will be deployed in each of up to 3 sections of the harbour. The sections should have either industrial (not fishing) boats, yachting/recreational (not fishing), and fishing vessels. This gives a maximum deployment of 3 gillnets and 3 fyke-nets per harbour. Harbour with only 1 or 2 sections represented will only be sampled in these sections.

The gear will be deployed in the evening around 8 pm and retrieved around 6 am. At each harbour the site for deployment will be confirmed with the harbour master in advance of the survey.

At each fishing station the date, time of deployment, time of retrieval, GPS position gear type and depth will be registered.

All fish species caught in a gill-net or a fyke-net will be identified to species and all fish measured (total length) rounded down to the nearest cm. Species which are difficult to identify will be either photographed or kept frozen for later identification in the laboratory. Invasive species specimen will be photographed collectively in one sample. All specimen of invasive species will be frozen. The fish from each section will be worked up separately (maximum 3 per harbour). Any crabs caught in the gill-nets or fyke-nets will also be registered according to species and numbers.

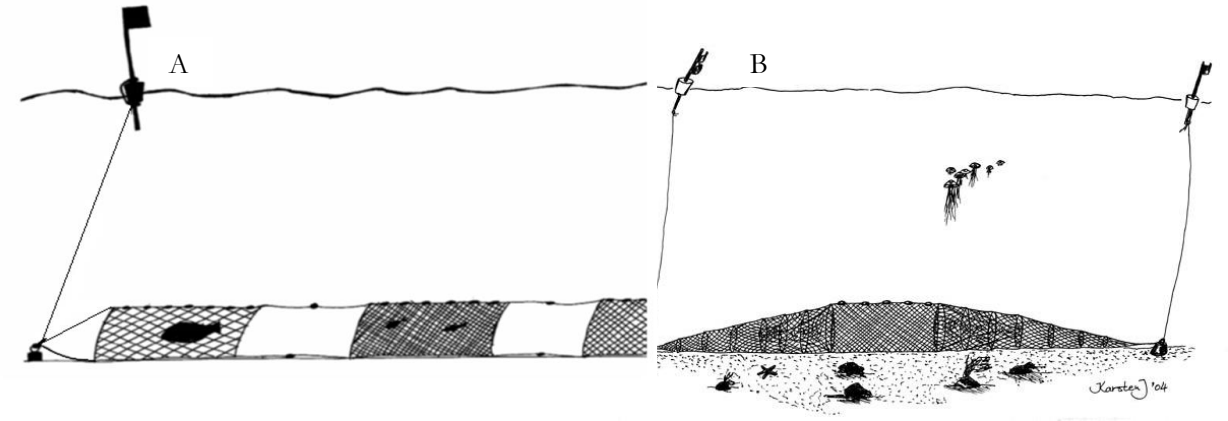

Figure 11*: A) Multi mesh gillnets (illustration by Hansen 2012 B) Fyke Nets. Illustation by http://dyk.nu/pages/net-typer.htm*

2.1.5 Fish, diving at night

As far as possible, snorkeling will be done along a pier or the like, along an approximately 500 m long transect (Figure 12) (see Sigsgaard et al. 2017). If the conditions inside the harbours make snorkeling impossible, for example due to poor visibility, snorkeling will be moved to the outer side of the pier. Initially, snorkeling is done in one direction in shallow water, near the pear. Thereafter, at a slightly deeper depth (up to 5 m) in the opposite direction. During these investigations, fish both associated with hard and soft bottom will be registered. The swimming speed will be adjusted so that the entire investigation is completed in 1 hour.

Snorkeling will, when possible, be performed in the hours of darkness, but if the conditions turns out to be dangerous in the specific port, snorkeling should be attempted during daytime. The visibility will be estimated, so that "swept area" can be estimated and the number of fish can be presented as number / m2. Temperature and salinity are recorded.

All fish are counted and identified to species. Species which are difficult to identify will if possible be collected for safe species identification. If other non-resident organisms of e.g. algae, mussels and crabs are observed, they will be registered. A representative selection of the fauna will be photographed / filmed - the focus will be on non-resident species. Concurrently, macroalgae and NIS-invertebrates will be registered and collected for ID-verification.

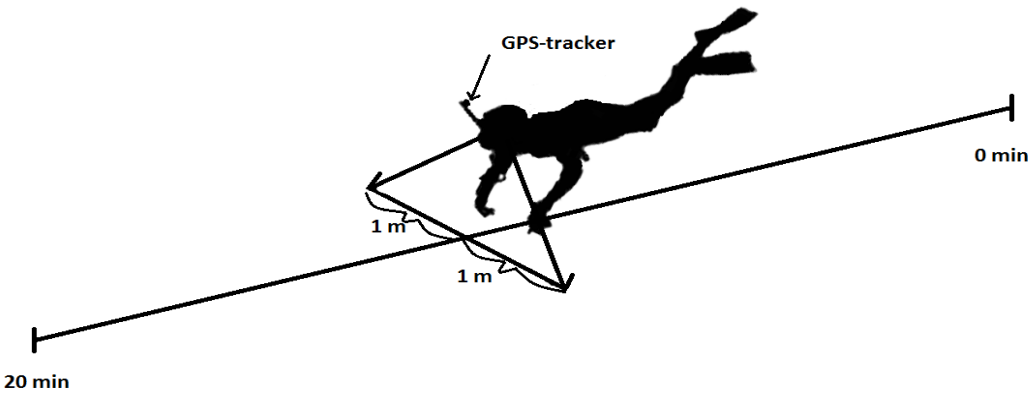

Figure 12: *Illustration of the diving approach with indicator of the surveyes area (Rasmus Ebert).*

2.2 Biomolecular methods

Environmental DNA (eDNA) will be sampled from 16 Danish harbours. The plan is to analyse for presence of DNA from 20 target species. Sampling and analysis of eDNA can be divided into three steps: Step 1: Sampling of water; step 2: filtration and storage, and step 3: DNA extraction and qPCR.

Equipment:

- Sample-kit with all single-use equipment needed (Amphi Consult sample-KitB)
- Amphiltrator (applied for pressure assisted filtration in the field).
- Dry ice for storage of samples.

Amphi Consult sample KitB includes: 500 ml Canister for water sampling, sample bag, funnel, luer-lock stoppers, 10 ml syringe, Sterivex-Filter, gloves and unique labels for labelling the samples (Figure 13).

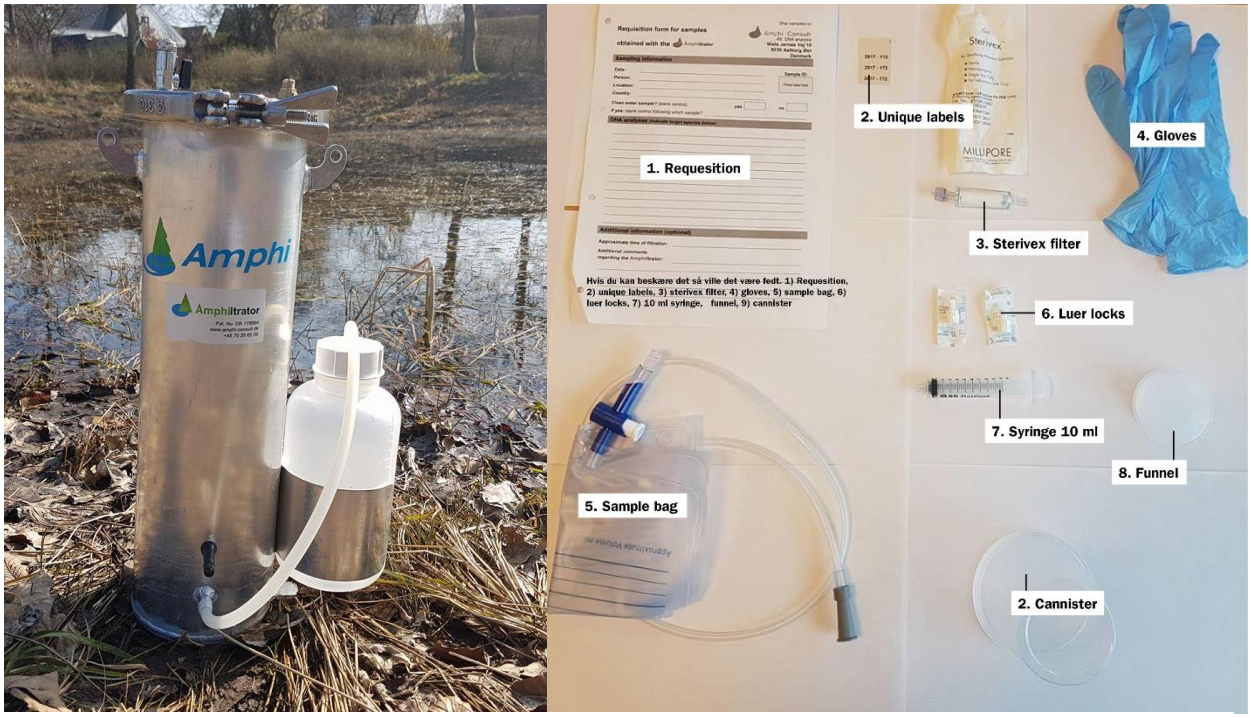

Figure 13: *Amphiltrator and sample KitB.*

locations are marked on the map as 1a and 1b, this will indicate that more distinct locations in the harbour are sampled. In that case water samples from 1a and 1b are mixed before filtration.

Sampling of water: The sampling of water will be carried out from the pier. The amount of eDNA is roughly proportional to the amount of water sampled. Therefore, the amount of water filtered (>1,5 l) will be maximised and larger particles in the collected water (i.e. macroalgae and insects) will be avoided. Sampling of water is done using a water sampler with a single-use plastic canister or bag to fill the sample bag through a funnel (Figure 14). Samples are taken approximately 1 m below surface. Different studies have showed, that benthic species are registered by eDNA sampling in surface waters in shallow and coastal areas.
If conditions are not suitable for sampling e.g. due to suspended material, sampling will be carried out outside of the harbour pier. The sampling of eDNA will take place in June and in August/September. Sampling on two occasions has been chosen to increase the chance to detect all species, since some algae are expected to be more numerous in spring compared to autumn, while some fish species are found at shallow water during the warm months in early autumn.

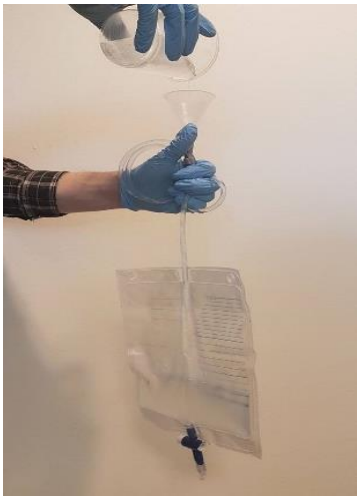

Figure 14: *Filling the sample bag.*

Filtration and storage:
Filtration of water with the Amphiltrator is done by attaching the 22 µm Sterivex filter to the Amphiltrator 2.0 and thereafter to the sample bag. The sample bag is then mounted and the Amphiltrator is safely closed by placing the O-ring, attaching the lid and clamp and tightening the screw according to the guidance. Then pressure is added to the chamber using a pump approx. 2.2 bar is needed for the filtration. (Figure 15). Filtration run until sample bag is emptied or to a suggested maximum of 30 minutes. The water temperature is measured along with the amount of filtrated water.

Figure 15: *Pressure added to the Amphiltrator.*

Figure 16: *After carefully emptying all water the Sterivex filter is labelled with unique label, luer-lock stoppers attached and stored on dry ice.*

Last step is opening the Amphiltrator and detaching the Sterivex filter. When the Sterivex filter is detached the provided syringe from sample KitB is used to remove the remaining water. This is important to properly preserve the DNA and to avoid filter damage subjected to freezing water. When the Sterivex filter is emptied, and luer-lock stoppers attached, a unique label is added to the filter (Figure 16), and immediately stored on dry ice (the sooner the better). The filters are transferred to -20° C freezing facility as soon as possible. For long-term storage (months), the filters must be transferred to -80 °C freezing facility as soon as possible. For each harbour two filters are made, one for analysis under the auspices of the NIS project and one for archiving.

DNA extraction and qPCR:
DNA extraction is done by using DNeasy Blood & Tissue kit (Qiagen) as described in Knudsen *et al.* (2015). Extracted eDNA is best preserved below -15 °C and for long-term storage the concentration from the extracted eDNA is measured (i.e. Nanodrop or Qubit).

The species-specific qPCR-detection systems targeting 21 selected non-indigenous species are currently being developed. The plan is that a minimum of 20 species-specific detection systems will be applied on each sample.

In this project detection of species-specific eDNA using qPCR on each filter is done by analysing a minimum of three replicates for each target species. These replicates are analysed for presence/absence of eDNA from the target species. Hence, the expected number of analysis is minimum: 64 filters, 20 species, 3 replicates; in total 1920 analyses.

The results from the qPCR may be quantified from the included standards with known concentration of target DNA. This is only possible, if eDNA concentration in the samples are sufficiently high. From the volume of filtrated water, and the volume of elution buffer added, the amount of target species-specific eDNA in the original water sample may be calculated. This may give an indication of quantitative differences between the investigated sites. It is currently not possible to estimate the population size of a given species using this method.

For a more detailed description of eDNA sampling and analysis, see Knudsen *et al.* (2015), Amphi Consult (2017) or the following video about the sampling procedure applied: https://youtu.be/2BniniPEpTc

3 Sampling schedule

Sampling of water samples for eDNA, will be carried out in June/July in the 14 harbours listed in Figure 17.

MONTH	JUNE							JULY													
WEEK	26							27							28						
DATE	26	27	28	29	30	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Aalborg Portland					x																
Aalborg Havn				x																	
Fredericia										xx											
Frederikshavn			x																		
Gedser									x												
Grenaa										xx											
Helsingør																xx					
Hirtshals			x																		
Kalundborg								x													
Københavns havn														xx							
Køge Havn									xx												
Odense Havn																					x
Rødby Færgehavn									x												
Statoil-havnen (Kalundborg)							x														

Figure 17: *Suggested dates for eDNA sampling in 14 harbours during June/July. Every sample is marked with a cross x. If there are two cresses "xx", night diving will be carried out. Weekends are greyed out. We reserve the right to change the dates if weather conditions do not allow sampling.*

Sampling of water samples for eDNA (eD), sampling by night diving (ND) and sampling for shellfish and fish (F) will be carried out in the 14 harbours during August listed in Figure 18. The three different sampling procedures will be carried out with approximately 2-5 days in between as it is apparent from Figure 18.

MONTH	AUGUST																																									
WEEK	33												34																		35											
DATE	17			18			19			20			21			22			23			24			25			26			27			28			29			30			31		
SAMPLE TYPE	eD	ND	F	eD	ND	F	eD	ND	F	eD	ND	F	eD	ND	F	eD	ND	F	eD	ND	F	eD	ND	F	eD	ND	F	eD	ND	F	eD	ND	F	eD	ND	F	eD	ND	F	eD	ND	F	eD	ND	F
Aalborg Portland																																												
Aalborg Havn																																												
Frederica																																												
Frederikshavn																																												
Gedser																			x	x															**x**			**x**							
Grenaa																																												
Helsingør																x	x				**x**			x																				
Hirtshals																																												
Kalundborg																																												**x**
Københavns havn				x	x										x			x																									
Køge Havn													x	x				**x**			x																							
Odense Havn																																											x	x	
Rødby Færgehavn																																			x	x				**x**			x			
Statoil-havnen (Kalundborg)																																									x	x				**x**

Figure 18: *Suggested dates for sampling in 14 harbours during August. Every sample is marked with a cross x. Type of sampling is indicated by the abbreviations* ***eD*** *for eDNA sampling,* ***ND*** *for Night Diving,* ***F*** *for sampling of Fish. Weekends are greyed out. We reserve the right to change the dates if weather conditions do not allow sampling.*

Sampling of water samples for eDNA (eD), sampling by night diving (ND) and sampling for shellfish and fish (F) will be carried out in the 14 harbours during September listed in Figure 19. The three different sampling procedures will be carried out with approximately 2-5 days in between as it is apparent from Figure 19.

MONTH	SEPTEMBER																																												
WEEK	35									36																					37														
DATE	1			2			3			4			5			6			7			8			9			10			11			12			13			14			15		
SAMPLE TYPE	eD	ND	F	eD	ND	F	eD	ND	F	eD	ND	F	eD	ND	F	eD	ND	F	eD	ND	F	eD	ND	F	eD	ND	F	eD	ND	F	eD	ND	F	eD	ND	F	eD	ND	F	eD	ND	F	eD	ND	F
Aalborg Portland																x	x																**x**			x									
Aalborg Havn																															x	x				x			x						
Fredericia										x	x				**x**			x																											
Frederikshavn																																		x	x				x			x			
Gedser																																													
Grenaa													x	x																x			x												
Helsingør																																													
Hirtshals																																					x	x				x			x
Kalundborg																																													
Københavns havn																																													
Køge Havn																																													
Odense Havn												**x**			x																														
Rødby Færgehavn																																													
Statoil-havnen (Kalundborg)			x																																										

Figure 19: *Suggested dates for sampling in 14 harbours during September. Every sample is marked with a cross x. Type of sampling is indicated by the abbreviations* ***eD*** *for eDNA sampling,* ***ND*** *for Night Diving,* ***F*** *for sampling of Fish. Weekends are greyed out. We reserve the right to change the dates if weather conditions do not allow sampling.*

Sampling of phytoplankton, zooplankton, settlement plates, benthic invertebrates, vegetation, water samples for eDNA, sampling by night diving and sampling for shellfish and fish will be carried out in the Aarhus and Esbjerg harbour at the dates shown in Figure 20.

AARHUS						
MONTH	***June***	***July***	**September**			
WEEK	***25***	***27***	*36*	*37*	*38*	*39*
Phytoplankton	21. June				21.-30. September	21.-30. September
Zooplankton	21. June				21.-30. September	21.-30. September
Settlement plates being immersed	21. June					
Settlement plates takes up					21.-30. September	21.-30. September
Benthic invertebrates					21.-30. September	21.-30. September
Vegetation					21.-30. September	21.-30. September
eDNA		5. July	5-6 September			
Night diving		5. July	5-6 September			
Fish and shellfish			7-8 September			

ESBJERG						
MONTH	***June***	***June***	**September**			
WEEK	***25-26***	***27***	*36*	*37*	*38*	*39*
Phytoplankton	20. June				21.-30. September	21.-30. September
Zooplankton	20. June				21.-30. September	21.-30. September
Settlement plates being immersed	20. June					
Settlement plates takes up					21.-30. September	21.-30. September
Benthic invertebrates					21.-30. September	21.-30. September
Vegetation					21.-30. September	21.-30. September
eDNA	27. June		4-5 September			
Night diving			4-5 September			
Fish and shellfish			6-7 September			

Figure 20: *Suggested dates for sampling in Esbjerg and Aarhus Harbour in June/July and September. We reserve the right to change the dates if weather conditions do not allow sampling.*

4 Next steps

The conventional sampling as well as the biomolecular sampling will be carried out in June and August/September 2017.

The samples will be processed at the end of 2018 and beginning of 2018.

Reporting and data transfer will be completed by the end of June 2018.

The general schedule for preparation, sampling, data processing and reporting can be seen on the following Gantt chart:

MONIS 4 activities	**2017**												**2018**											
	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12
Kick-off and coordination meeting																								
Sampling protocol																								
Forårs-indsamling (plankton)[1]																								
Sommer/sen-sommer-indsamling																								
Sampling of water (eDNA)																								
Progress meeting 1																								
Oparbejdning (conventionel sampling)																								
Dataanalyser (conventional methods)																								
Lab tests og oparbejdning m.v. (eDNA)																								
Dataanalyser (eDNA)																								
Progress meeting 2																								
Reporting, draft																								
Reporting, final version																								

[1] *Sampling will include phytoplanton and zooplankton.*

NIVA Denmark and partners expect cf. above that the project will completed with reporting and data transfer the 30 June 2018.

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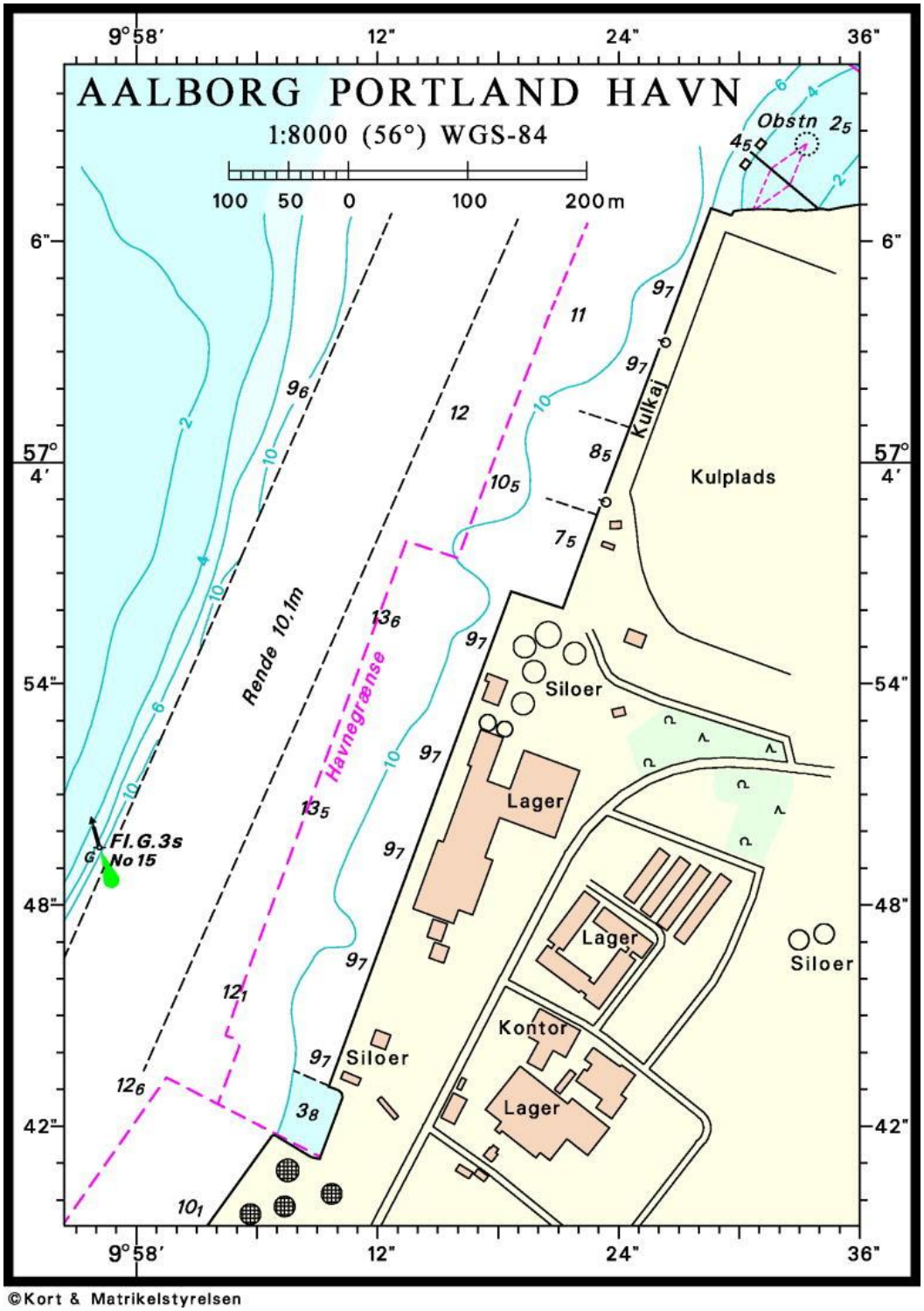
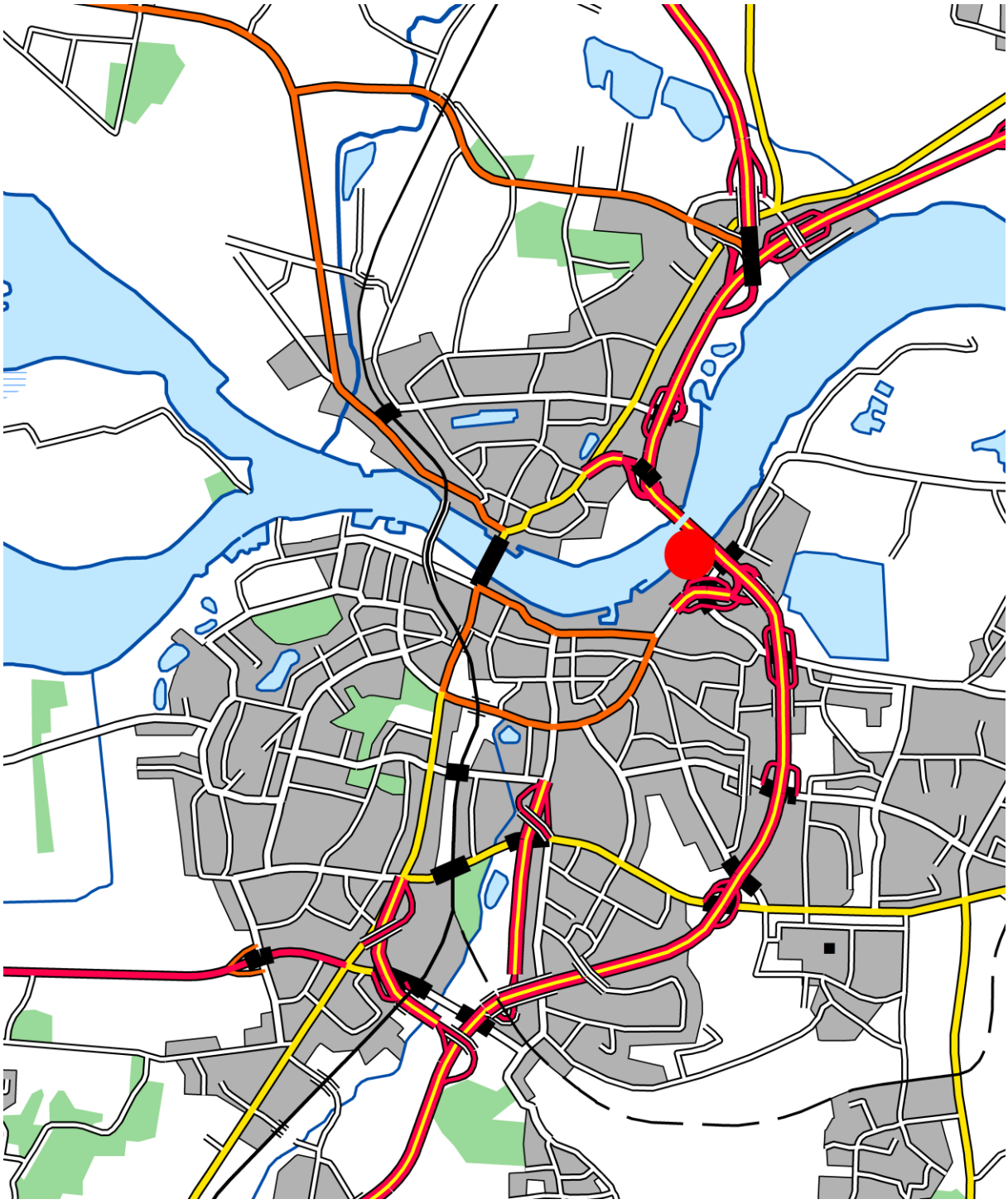
Annex 1: List of contacts

Harbour	Adresse	Telefon	Mail	Kontaktperson
Århus harbour	Vandvejen 7, 8000 Aarhus C	86 13 32 66	maritim@port.aarhus.dk	
Esbjerg harbour	Hulvejen 1, 6700 Esbjerg	76 12 40 00	vagt@portesbjerg.dk	
Aalborg Portland	Rørdalsvej 44, 9100 Aalborg	98 77 70 62	havn@aalborg-portland.dk	
Aalborg harbour	Langerak 19, 9220 Aalborg Øst	99 30 15 00	trafik@aalborghavn.dk	
Fredericia harbour	Centerhavnsvej 13, 7000 Fredericia	7921 5000	trafik-fh@adp-as.dk	
Frederikshavn harbour	Oliepieren 7, 9900 Frederikshavn	96 20 47 18	vagt@fhhavn.dk	
Gedser harbour	Havnegade 15, 4874 Gedser	21 76 23 75 (Ferry) 54 17 92 45 (Fishery)	pfso.gedser@scandlines.dk	
Grenaa harbour	Neptunvej 1, 8500 Grenaa	87 58 76 00	info@grenaahavn.dk	
Helsingør harbour	Nordhavnsvej 13, 3000 Helsingør	49 21 05 15	havnevagten@helsingor.dk	Jesper Schrøder
Hirtshals harbour	Norgeskajen 11, 9850 Hirtshals	98 94 14 22	havnevagten@hirtshalshavn.dk	
Kalundborg harbour	Baltic Plads, 4400 Kalundborg	59 53 40 00	info@portofkalundborg.dk	
Københavns harbour	Nordre Toldbod 7, 1259 København K	35 46 11 11	cmport@cmport.dk	
Køge harbour	Baltic Kaj 1, 4600 Køge	56 64 62 60	info@koegehavn.dk	
Odense harbour	Noatunvej 2, 5000 Odense C	72 28 20 10	havnekontor@odensehavn.dk	
Rødby Ferry harbour	Østre Kaj 3, 4970 Rødby	54 60 57 22	havne@lolland.dk	
Statoilhavnen (Kalundborg)		59 57 45 21	d01kapi@statoil.com	

Annex 2: Maps of individual harbours

3.1 Aalborg Portland harbour





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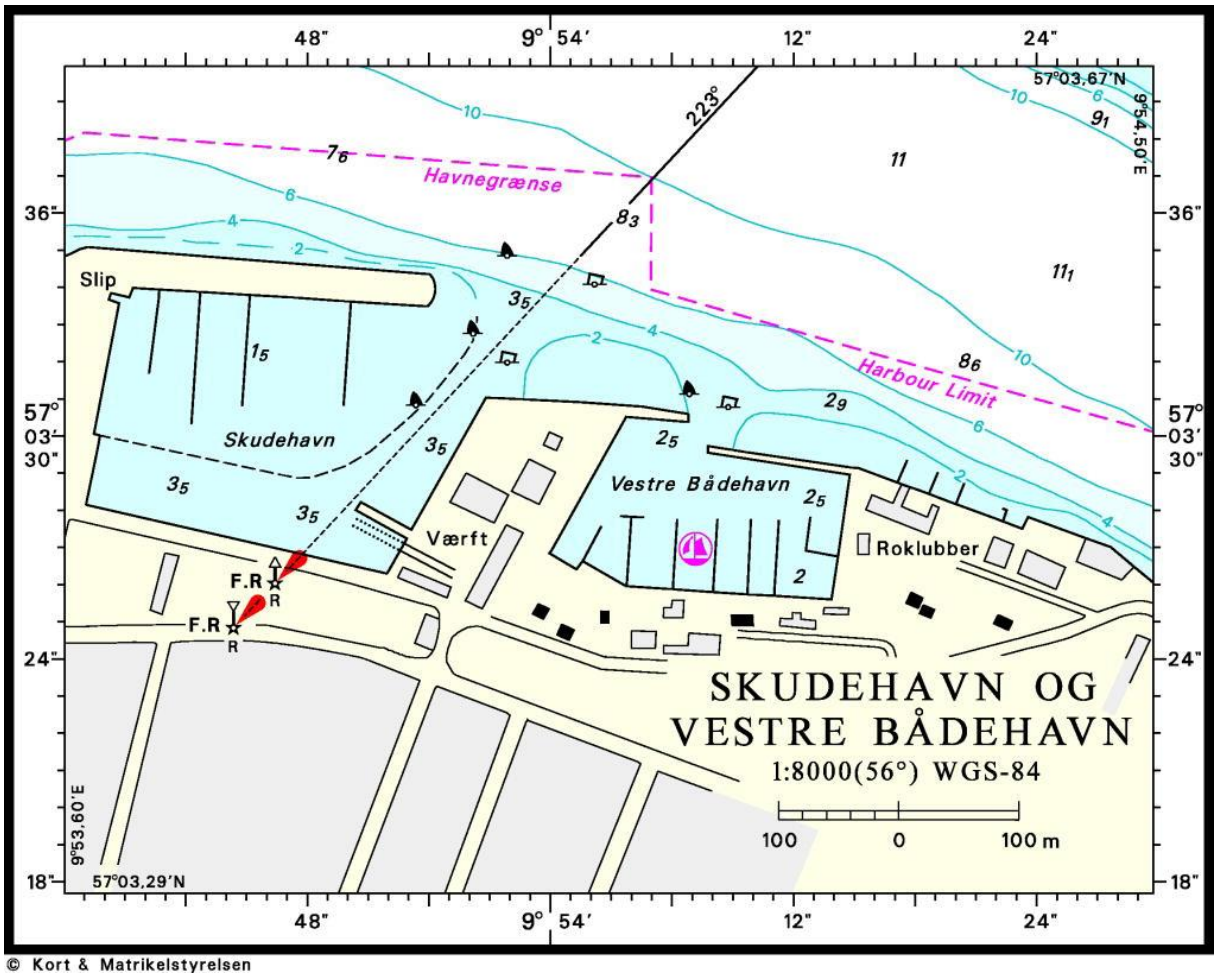
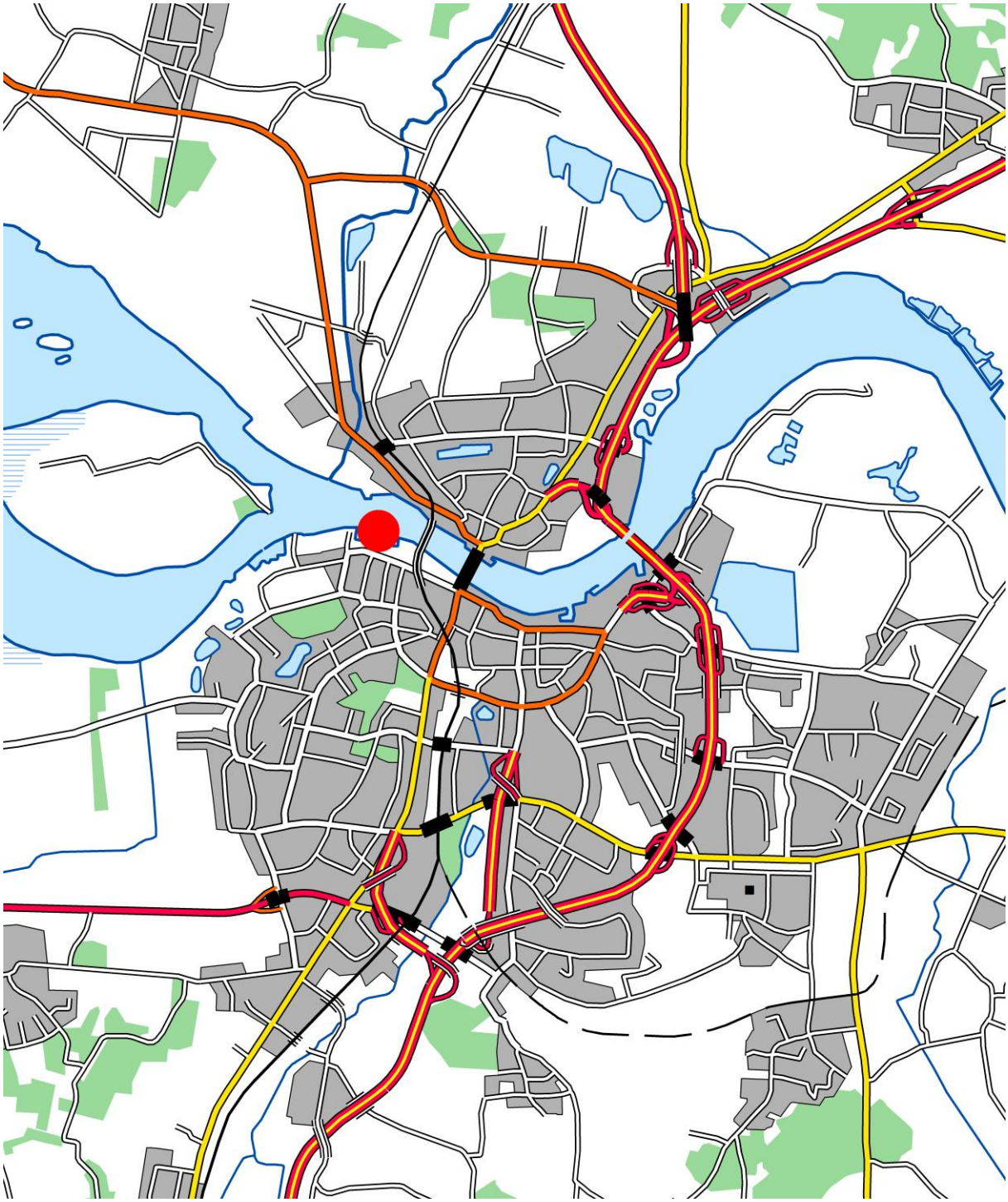
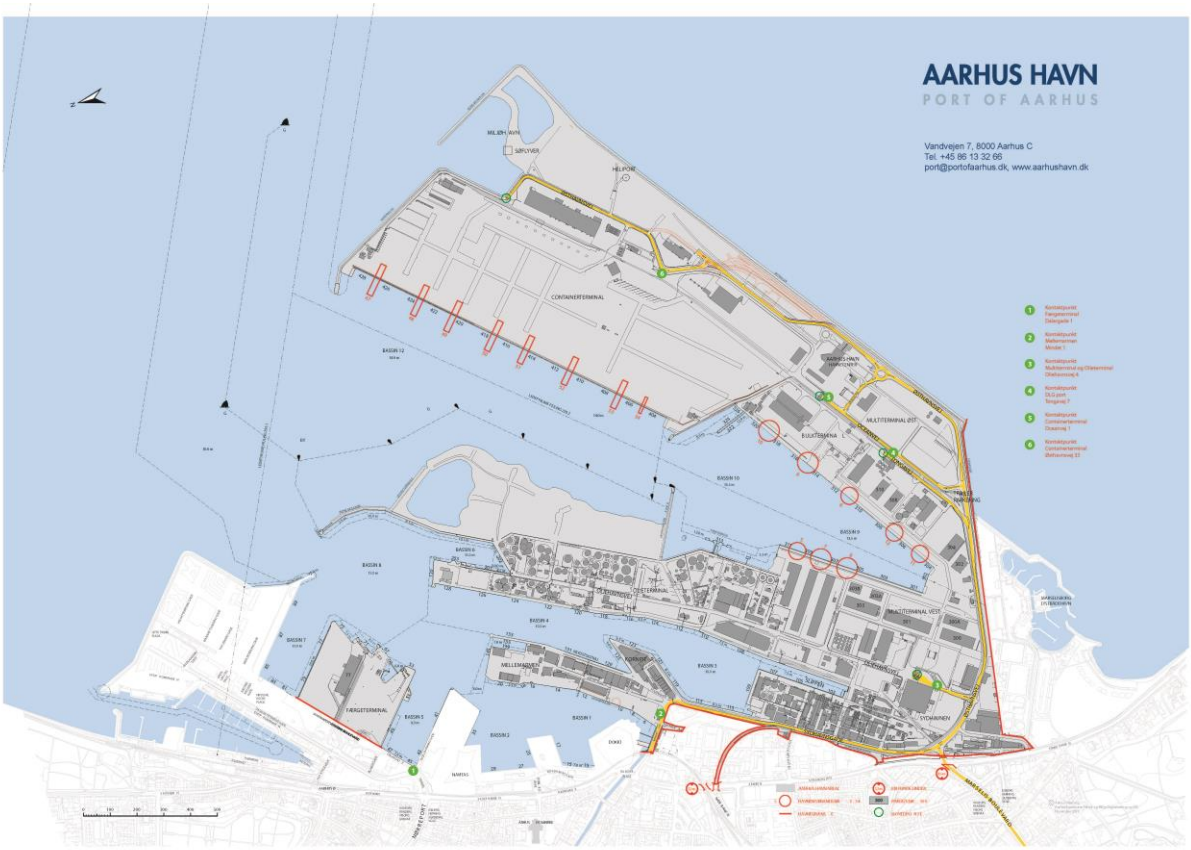
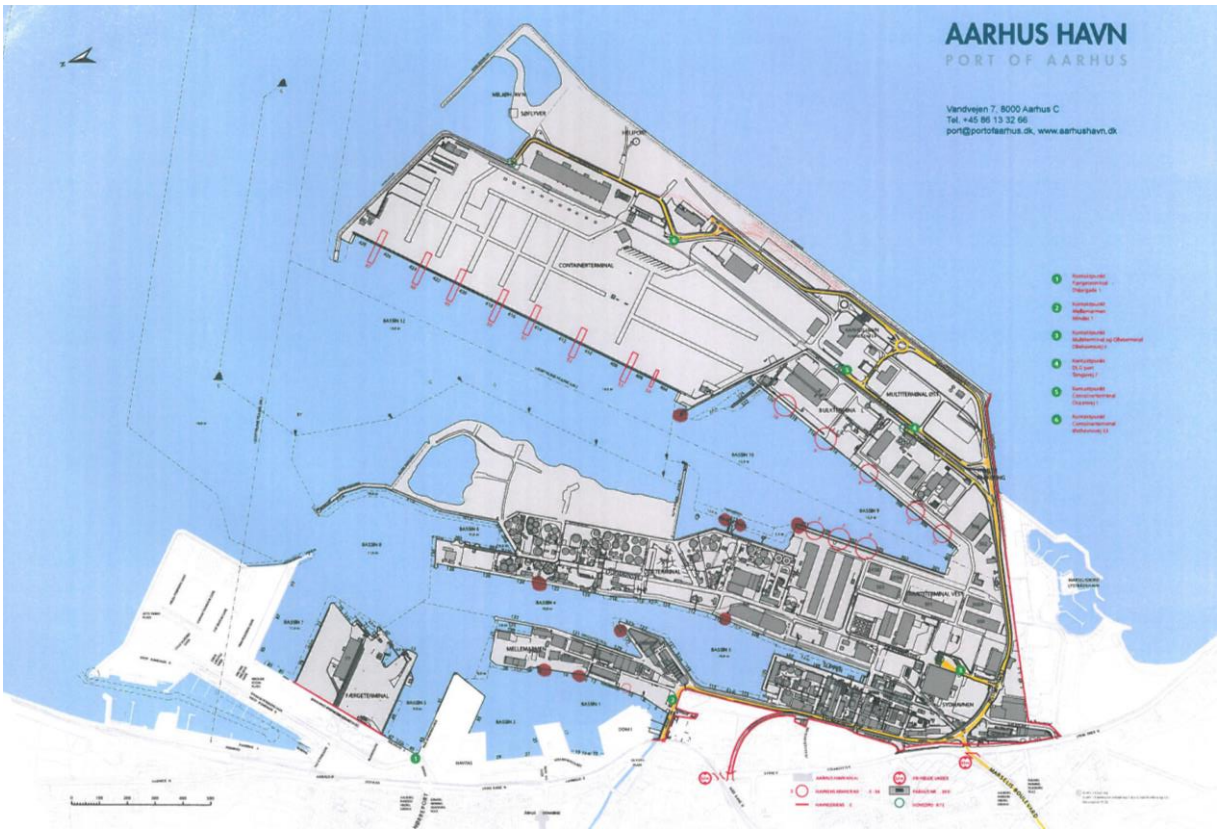


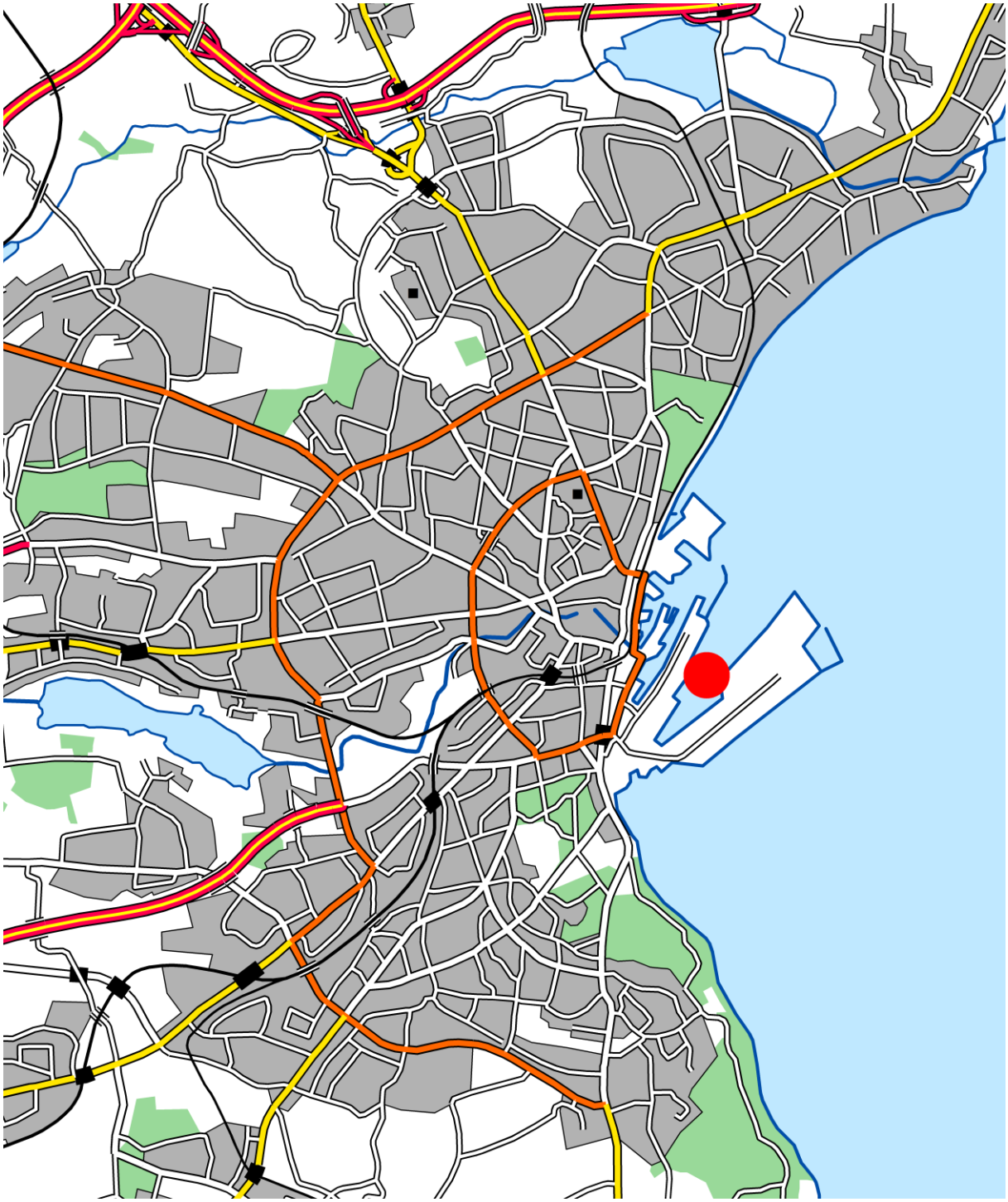


3.3 Aarhus


AARHUS HAVN
PORT OF AARHUS
Vandvejen 7, 8000 Aarhus C
Tel. +45 86 13 32 66
port@portofaarhus.dk, www.aarhushavn.dk

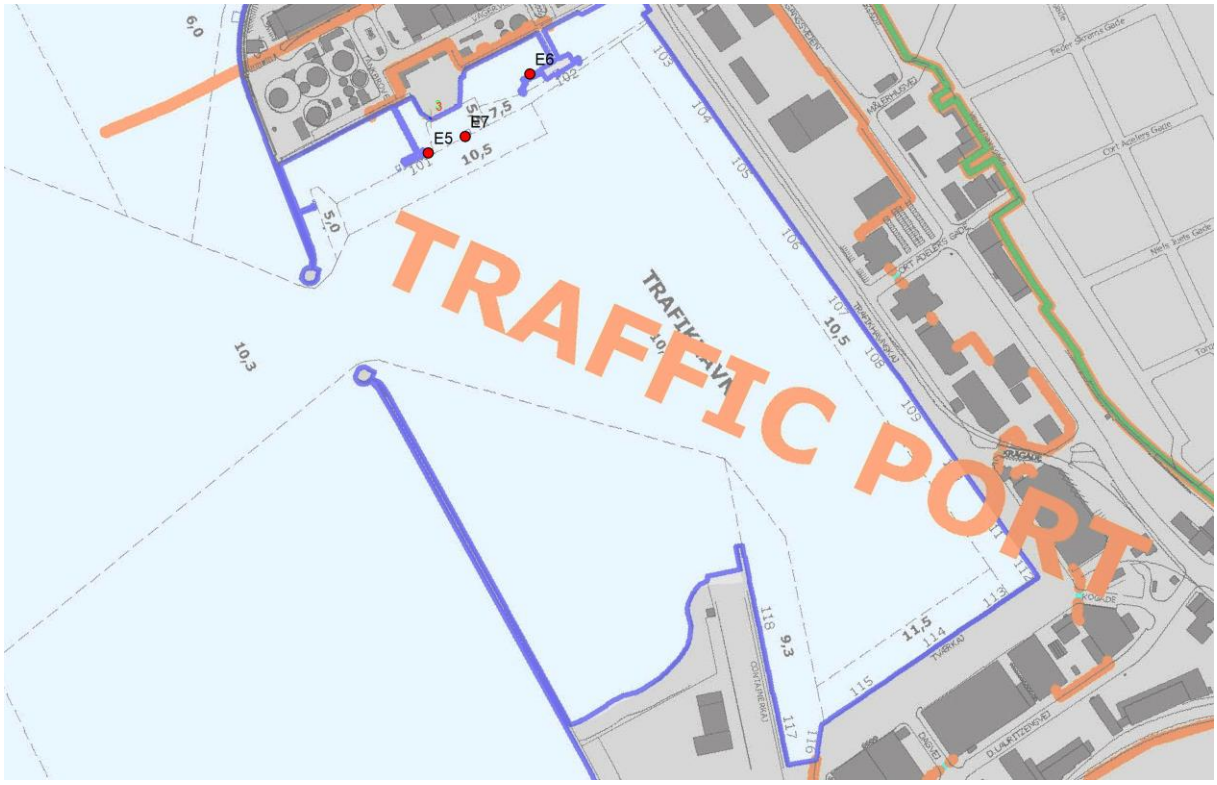

AARHUS HAVN
PORT OF AARHUS
Vandvejen 7, 8000 Aarhus C
Tel. +45 86 13 32 66
port@portofaarhus.dk, www.aarhushavn.dk


Å5
Å6
Å7
Å8
Å9
Å4
Å2Å3
Å1



3.4 Esbjerg


ESBJERG BEACH
NORTH PORT
E8
E9
E5
E7
TRAFFIC PORT
PORT SECTION
PORT SEA AREA
E4
E3
DOCK PORT
SOUTH PORT
EAST PORT
MAADE
E1E2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E7
E5
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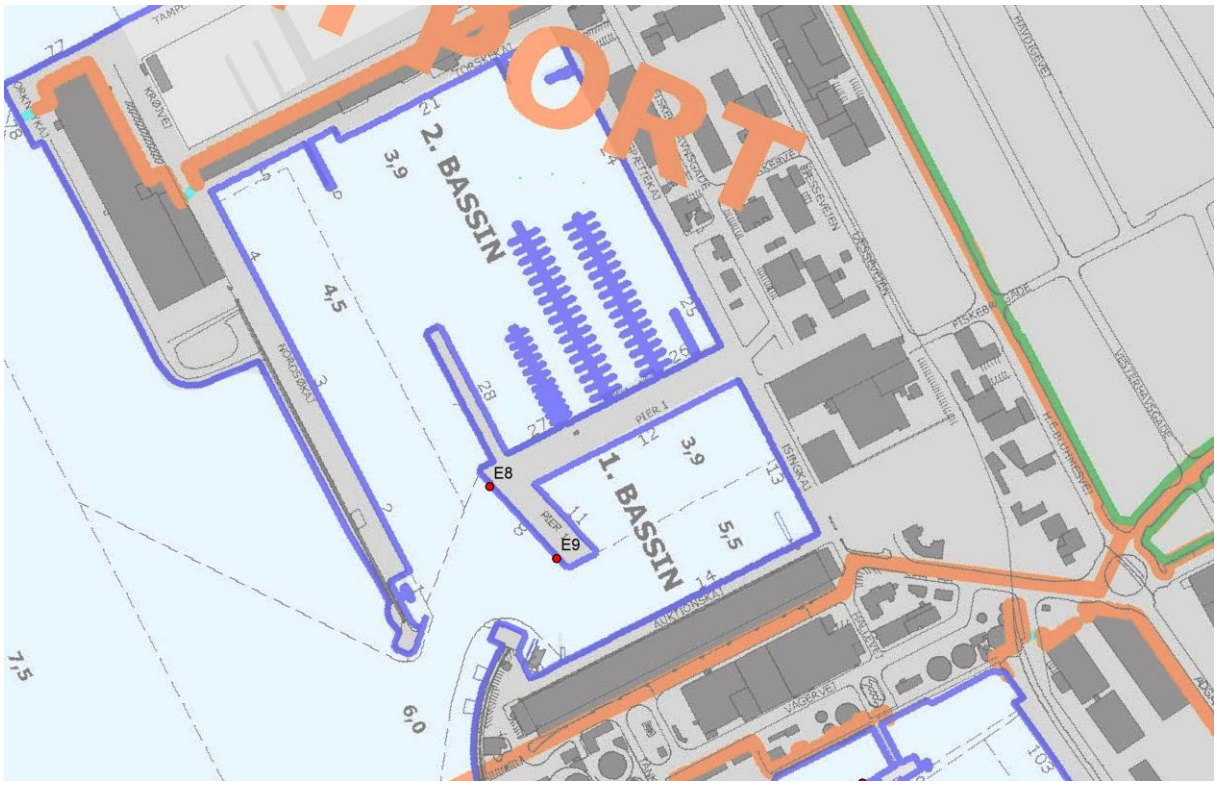
TRAFFIC PORT
10,5
9,3
11,5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1. BASSIN
E8
E9
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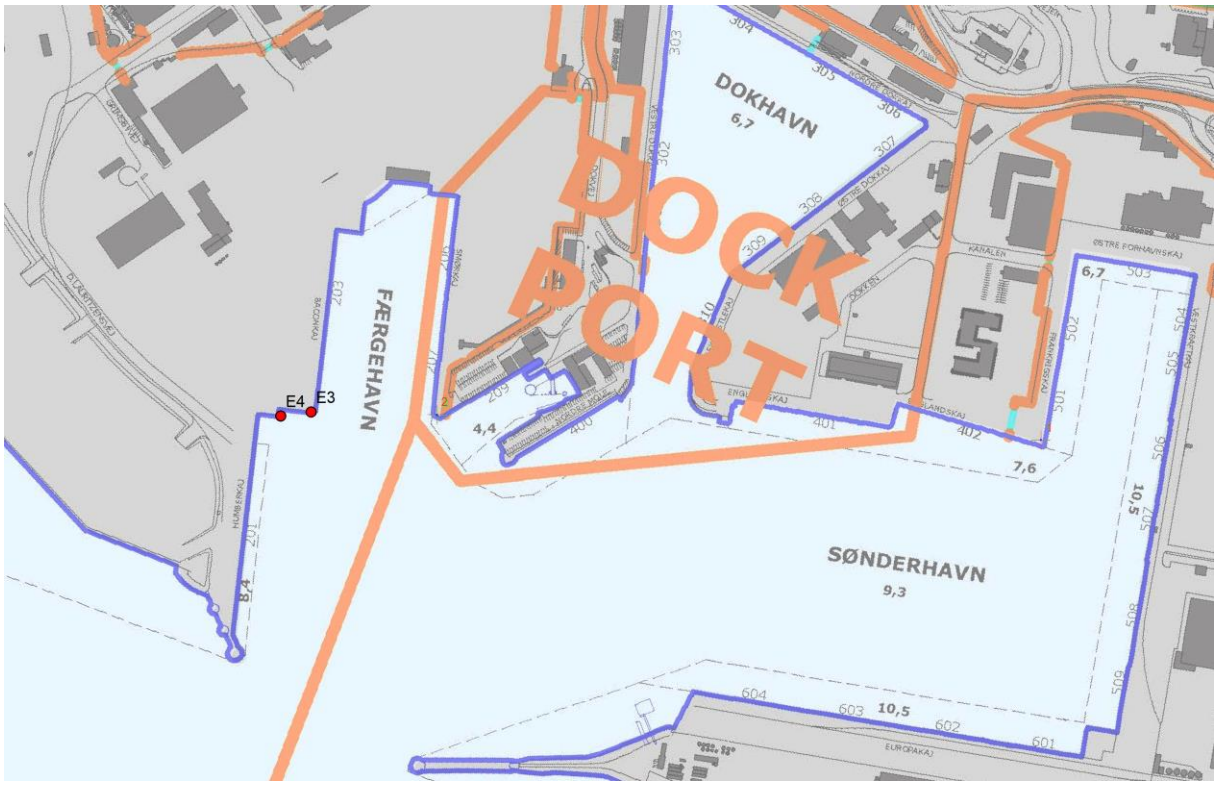
4,5
5,5
6,0
7,5


DOKHAVN
6,7
FÆRGEHAVN
SØNDERHAVN
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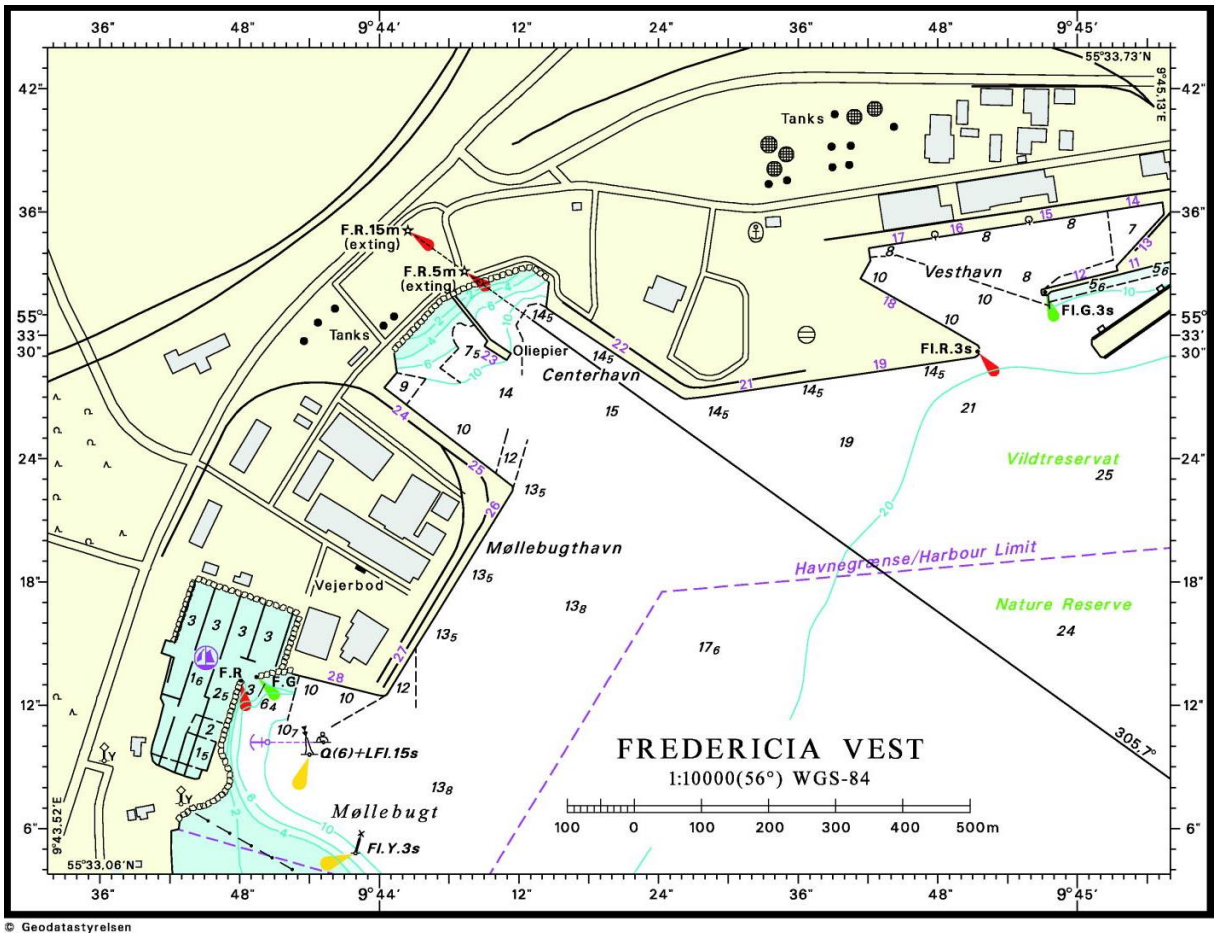
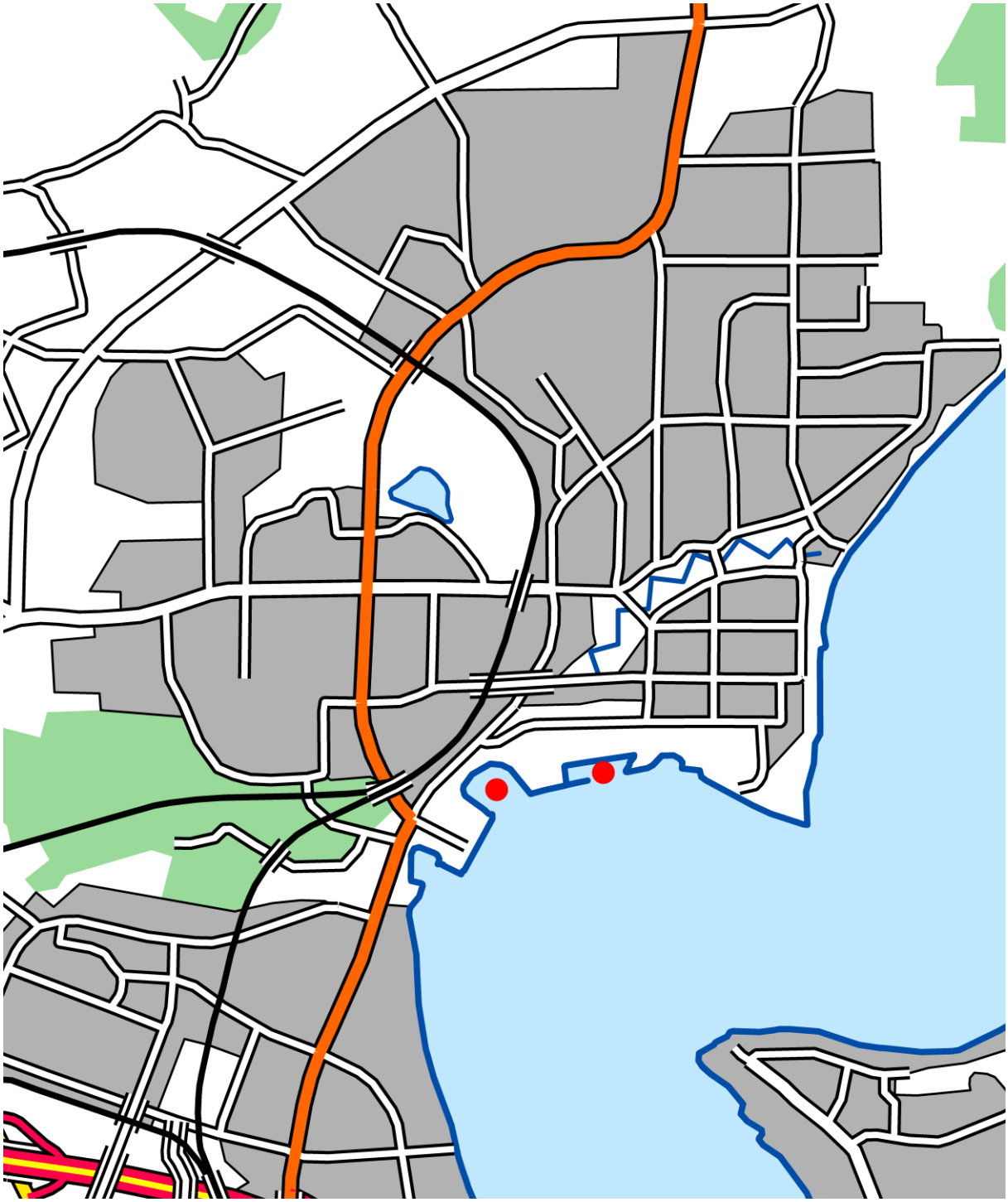
E4
E3
4,4
7,6
10,5
8,4


EAST PORT
Under Anlæg
E1E2

3.5 Fredericia





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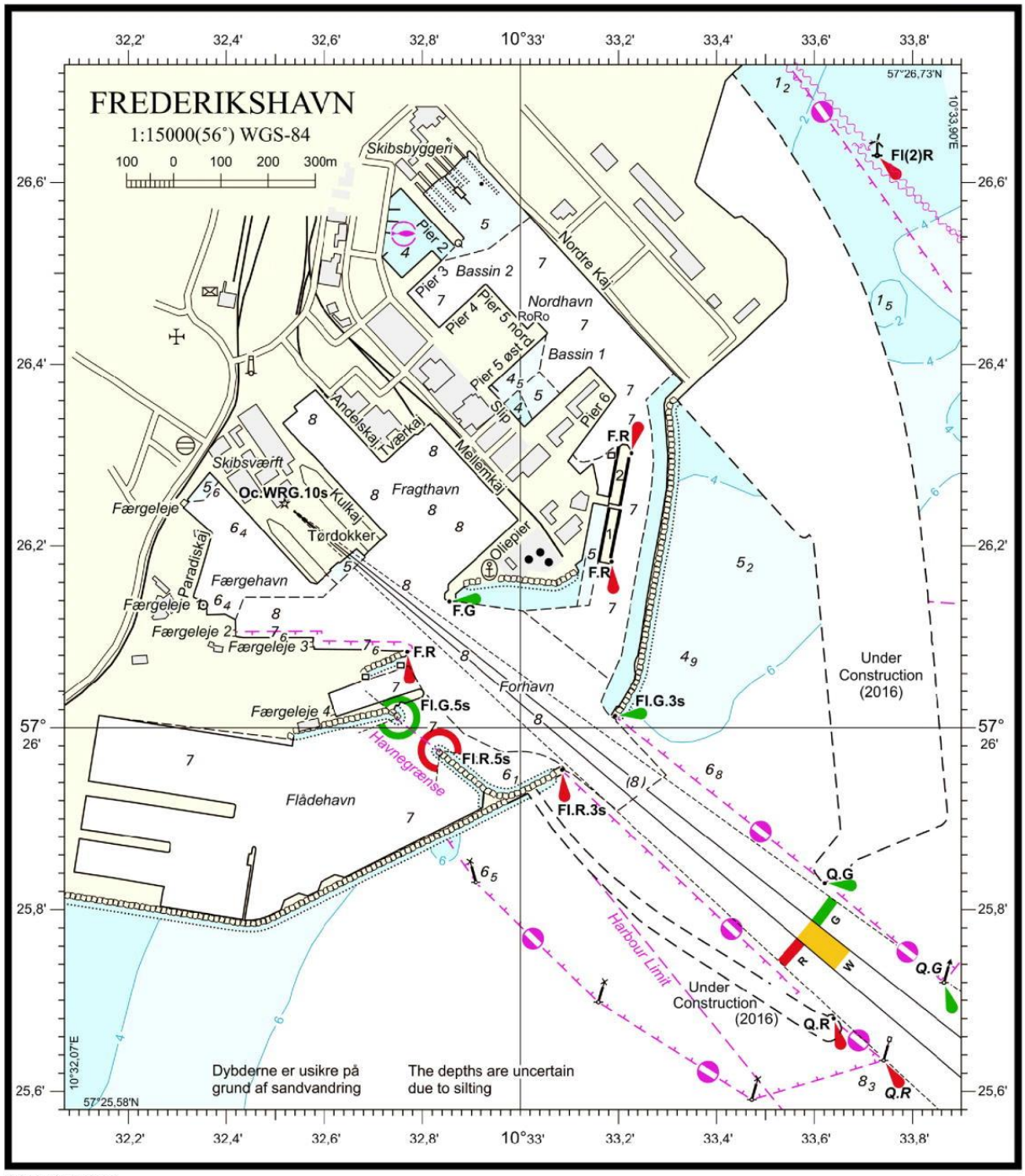

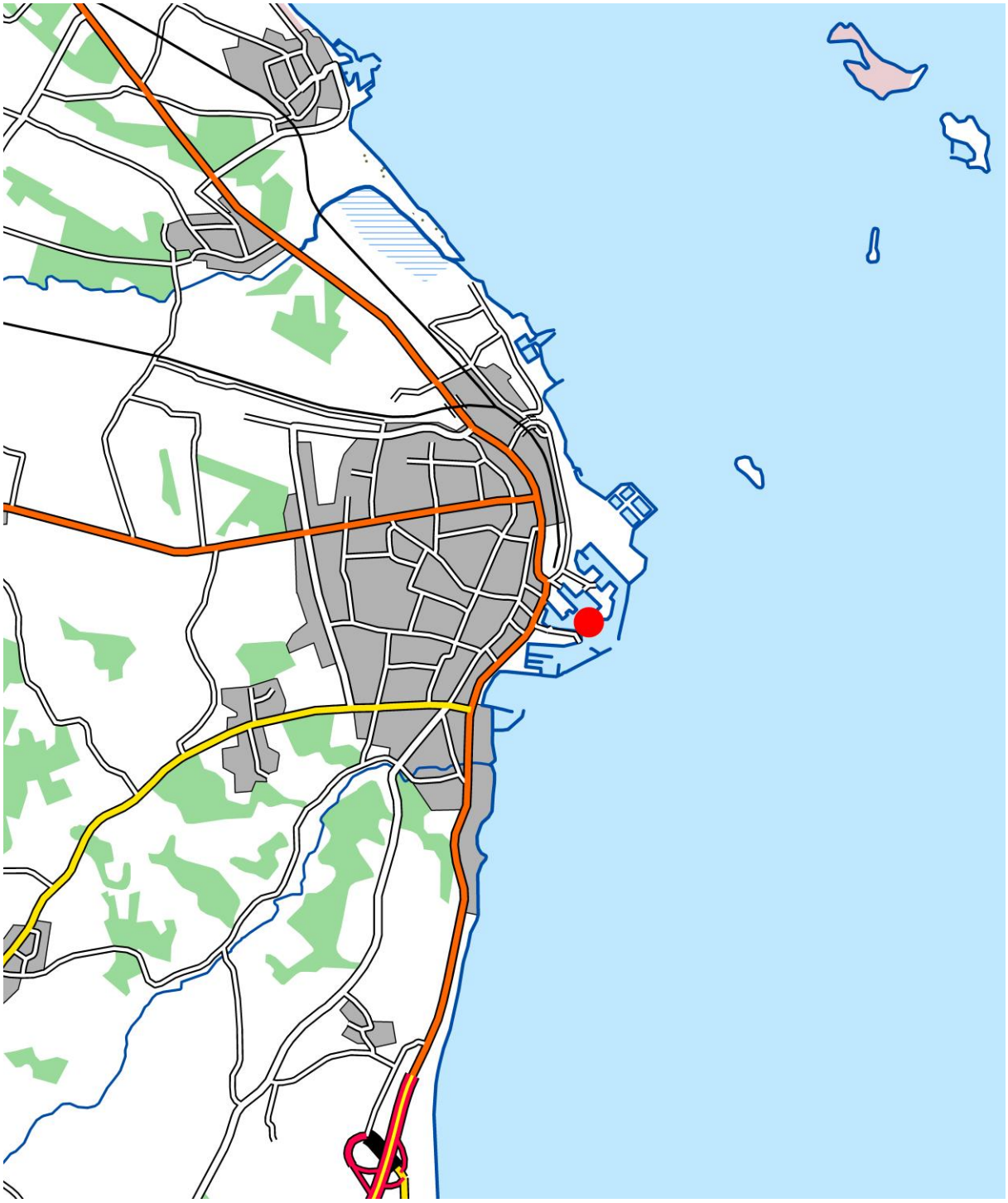

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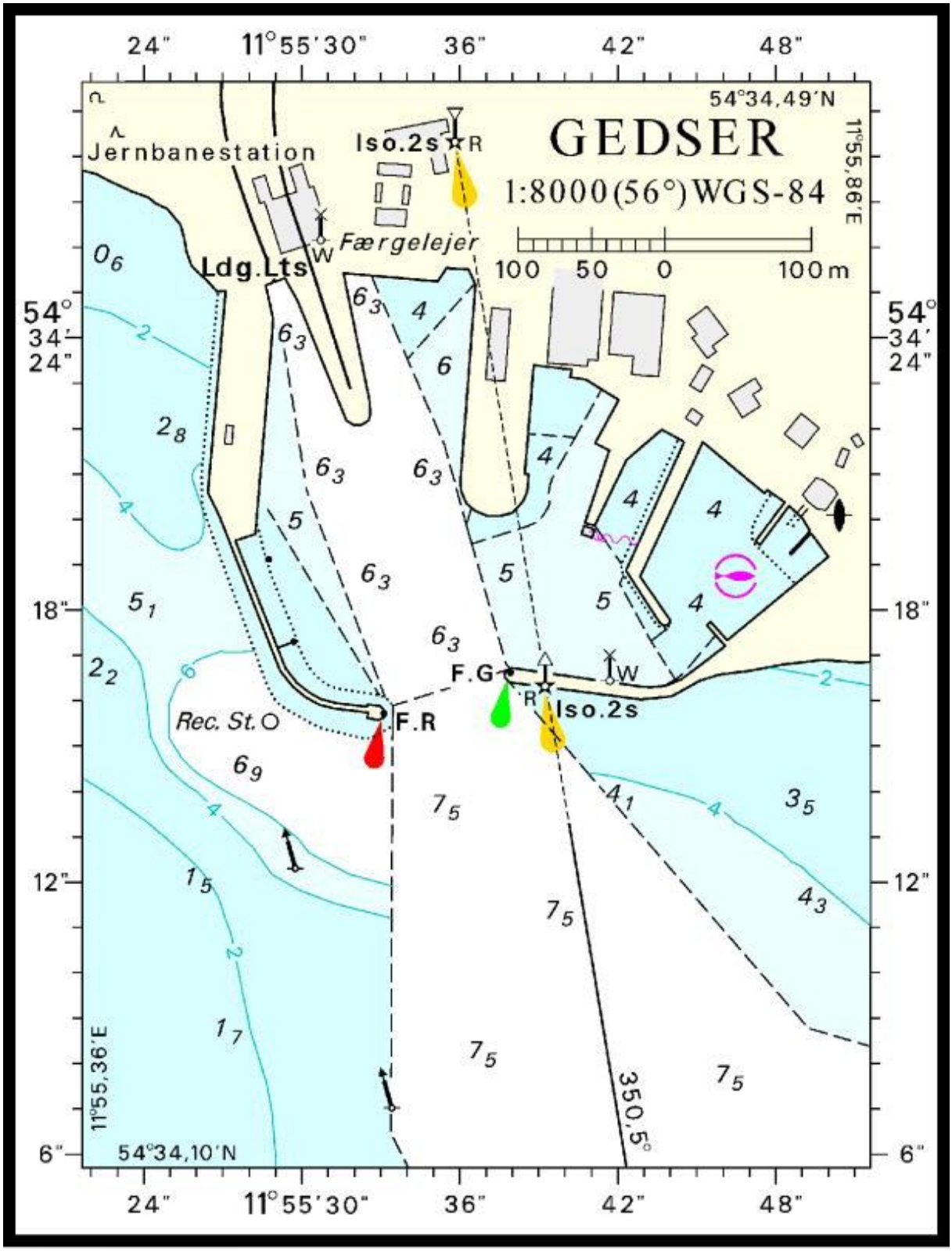
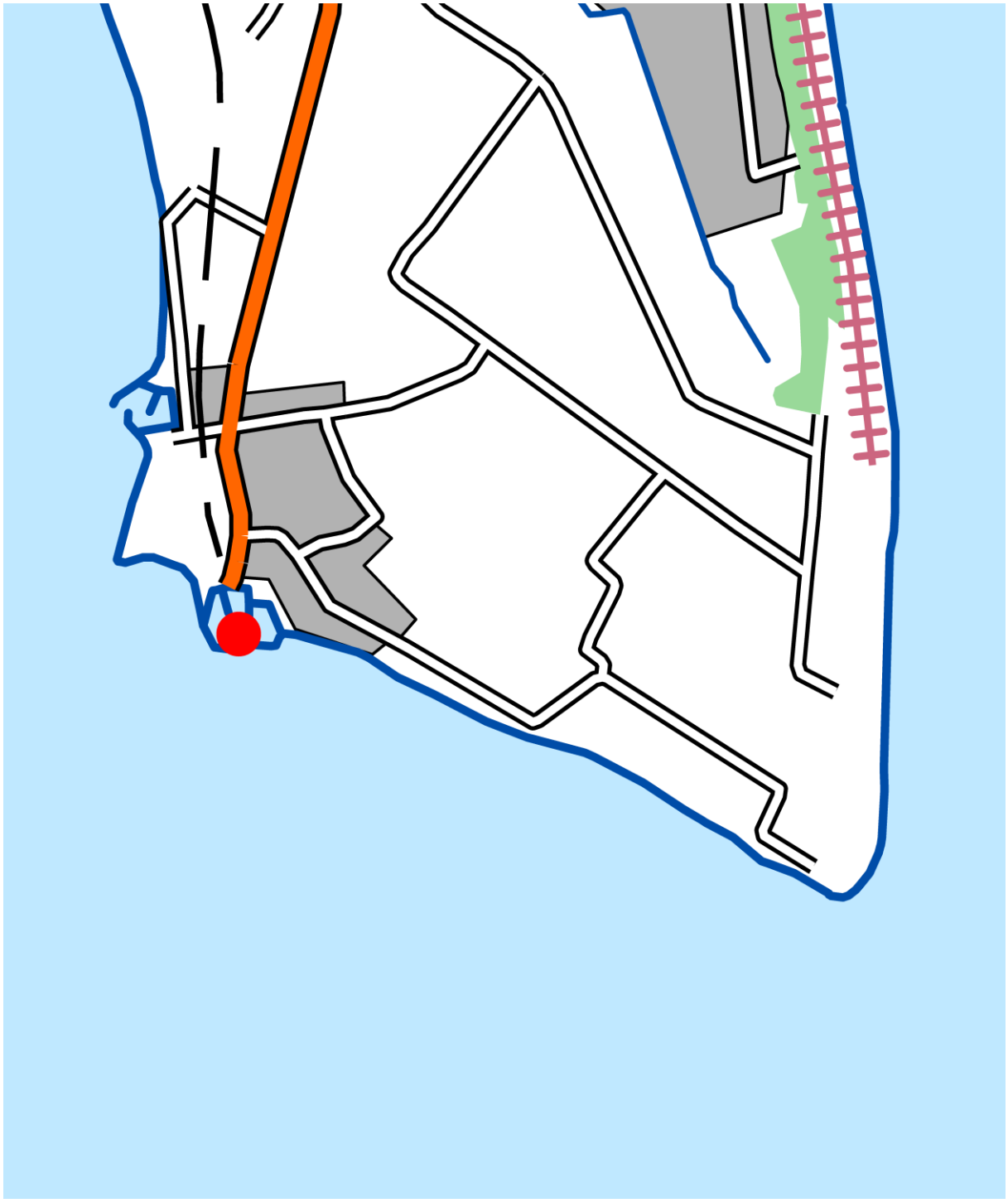
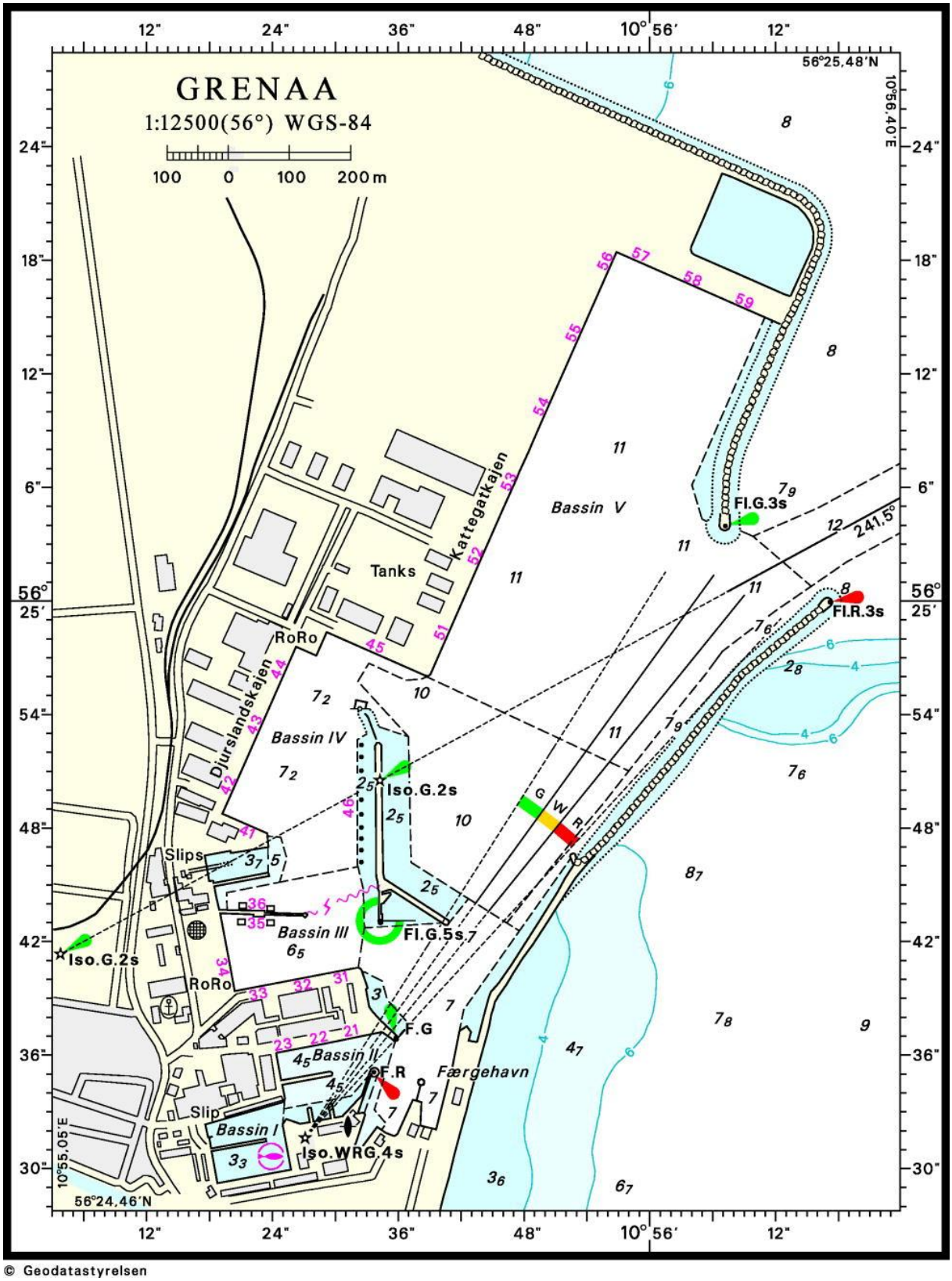




3.8 Grenaa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56°25,48'N
10°56,40'E
10°55,05'E
56°24,46'N
Bassin V
Bassin IV
Bassin III
Bassin II
Bassin I
Tanks
RoRo
RoRo
Slips
Slip
Kattegatkajen
Djurslandskajen
Færgehavn
Fl.G.3s
Fl.R.3s
Iso.G.2s
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F.R
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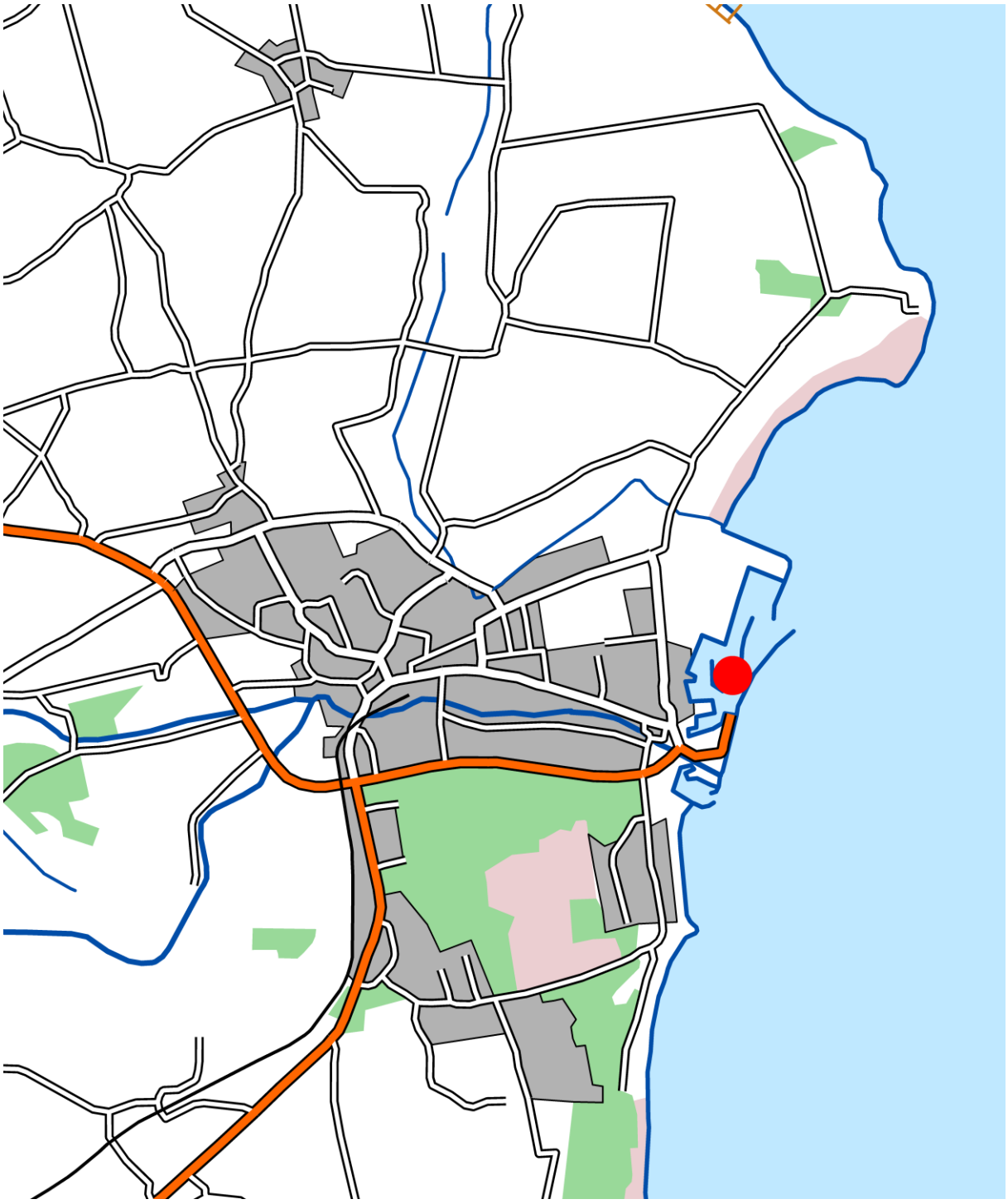
© Geodatastyrelsen



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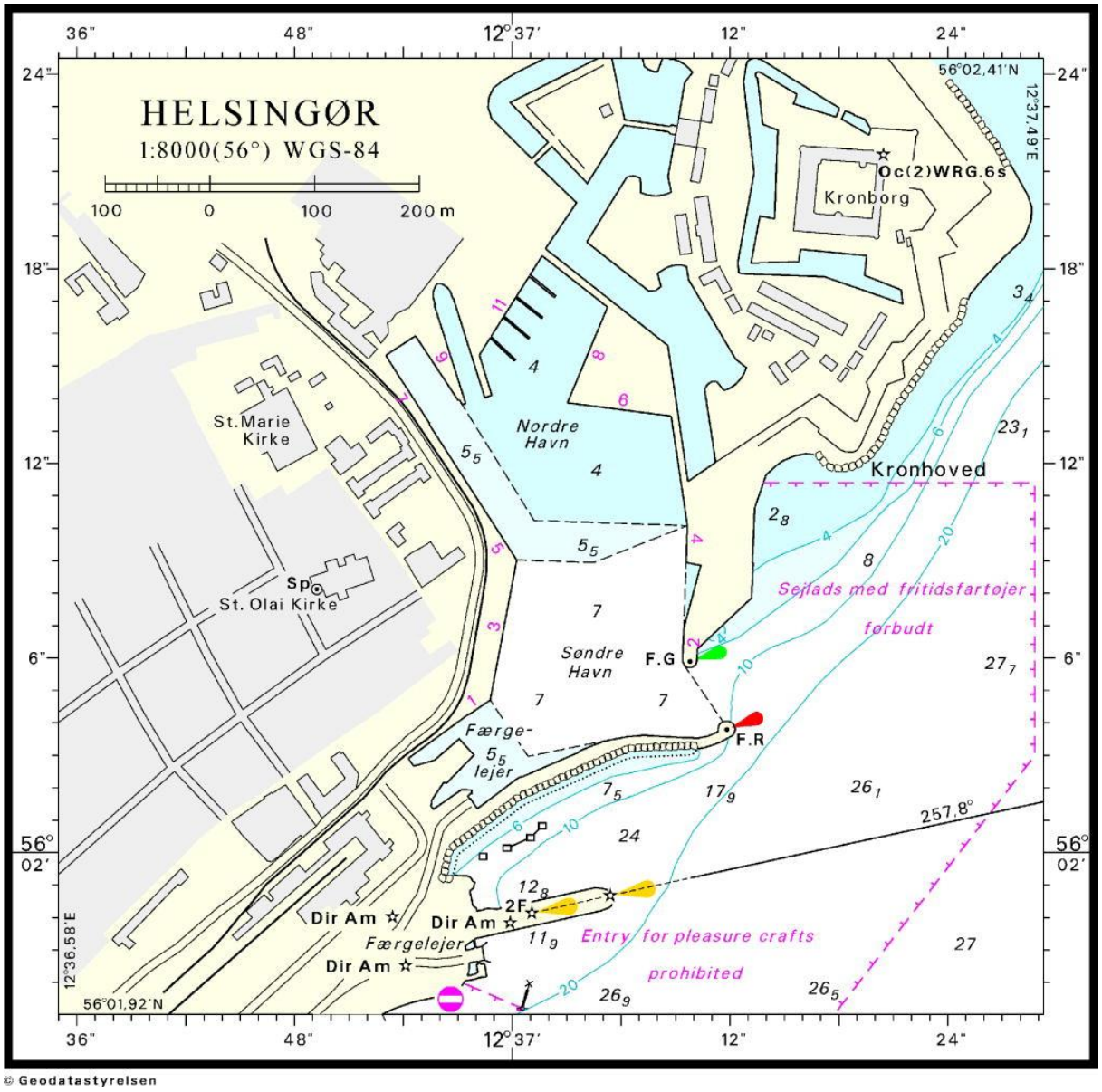

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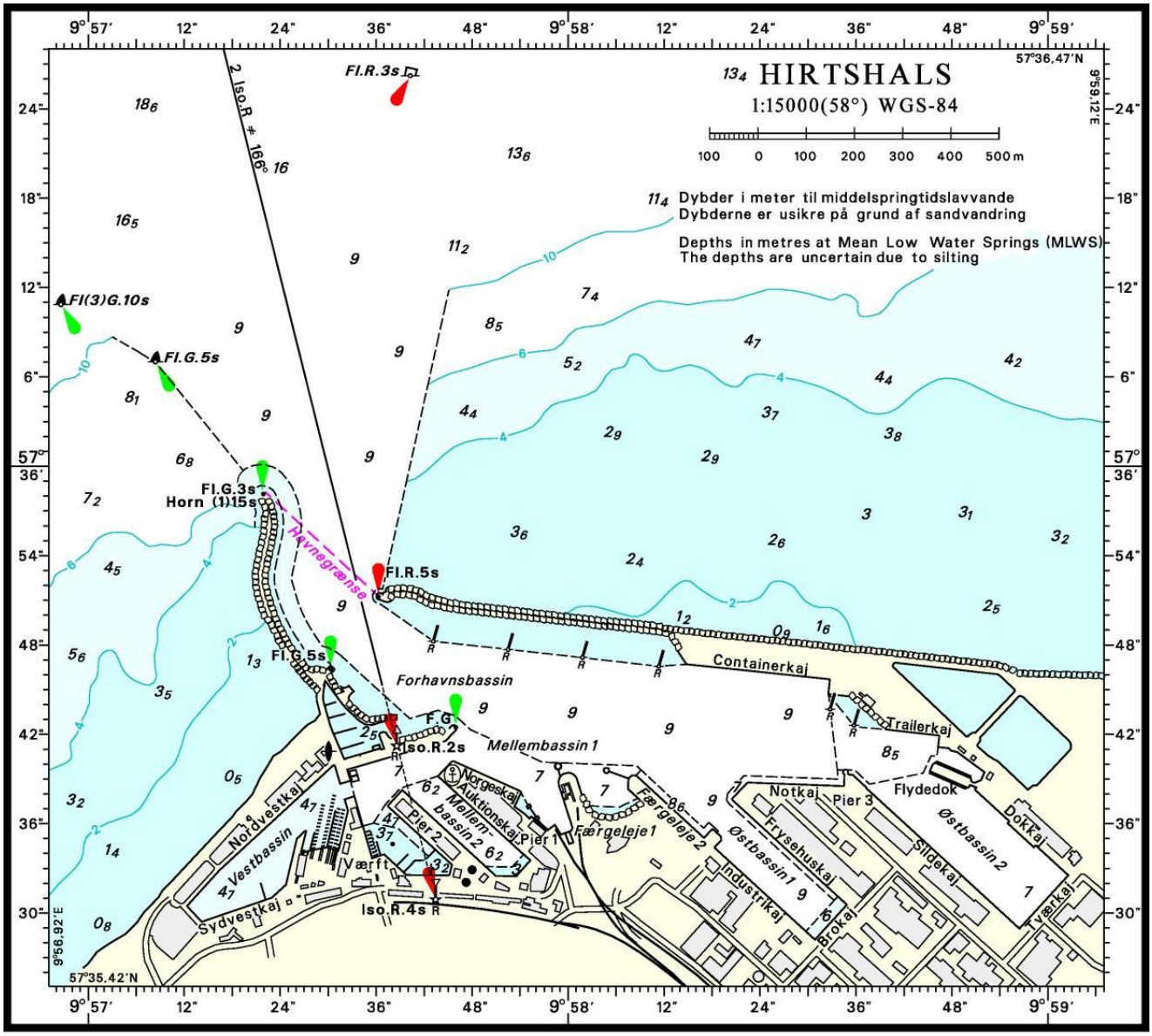

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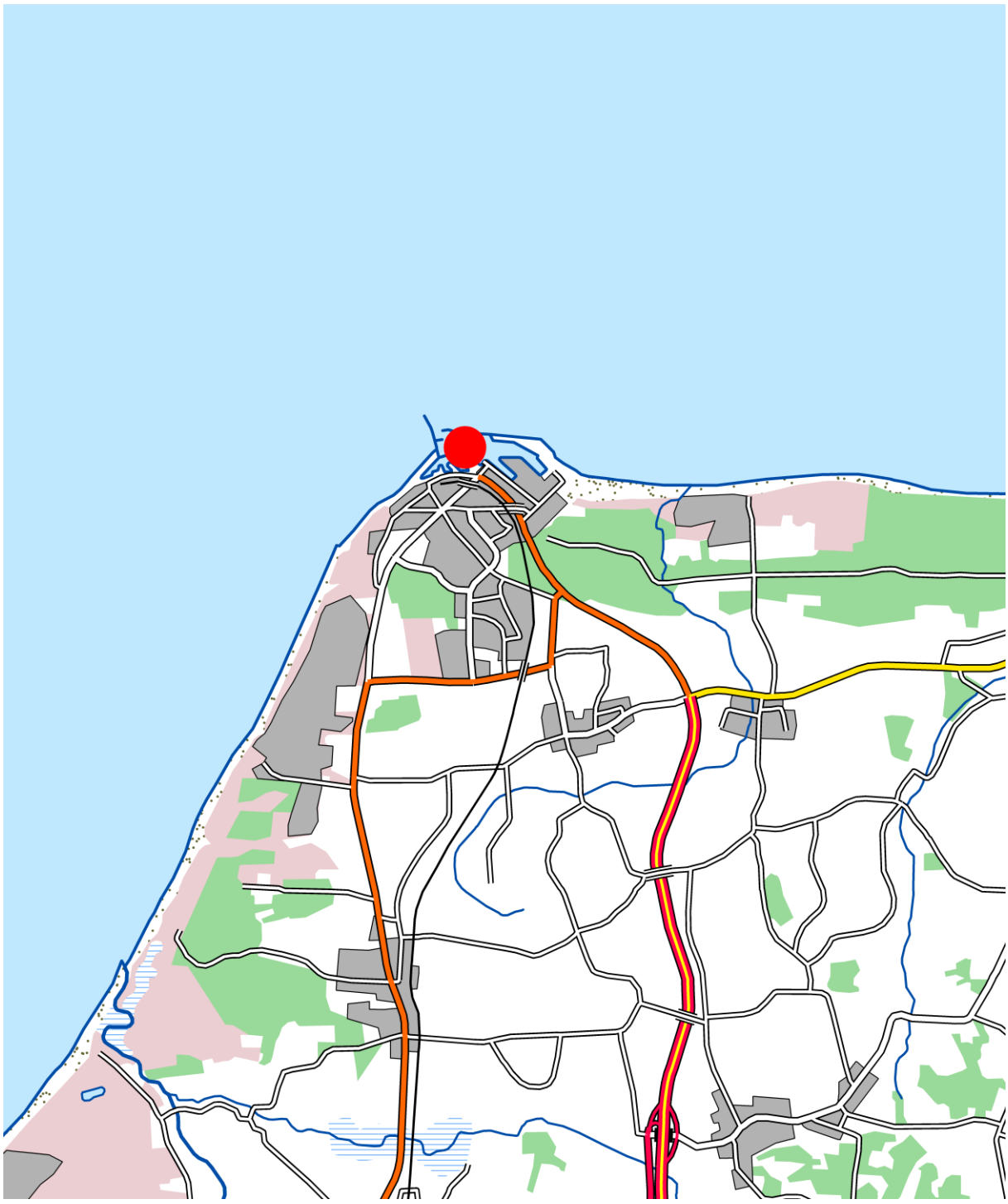

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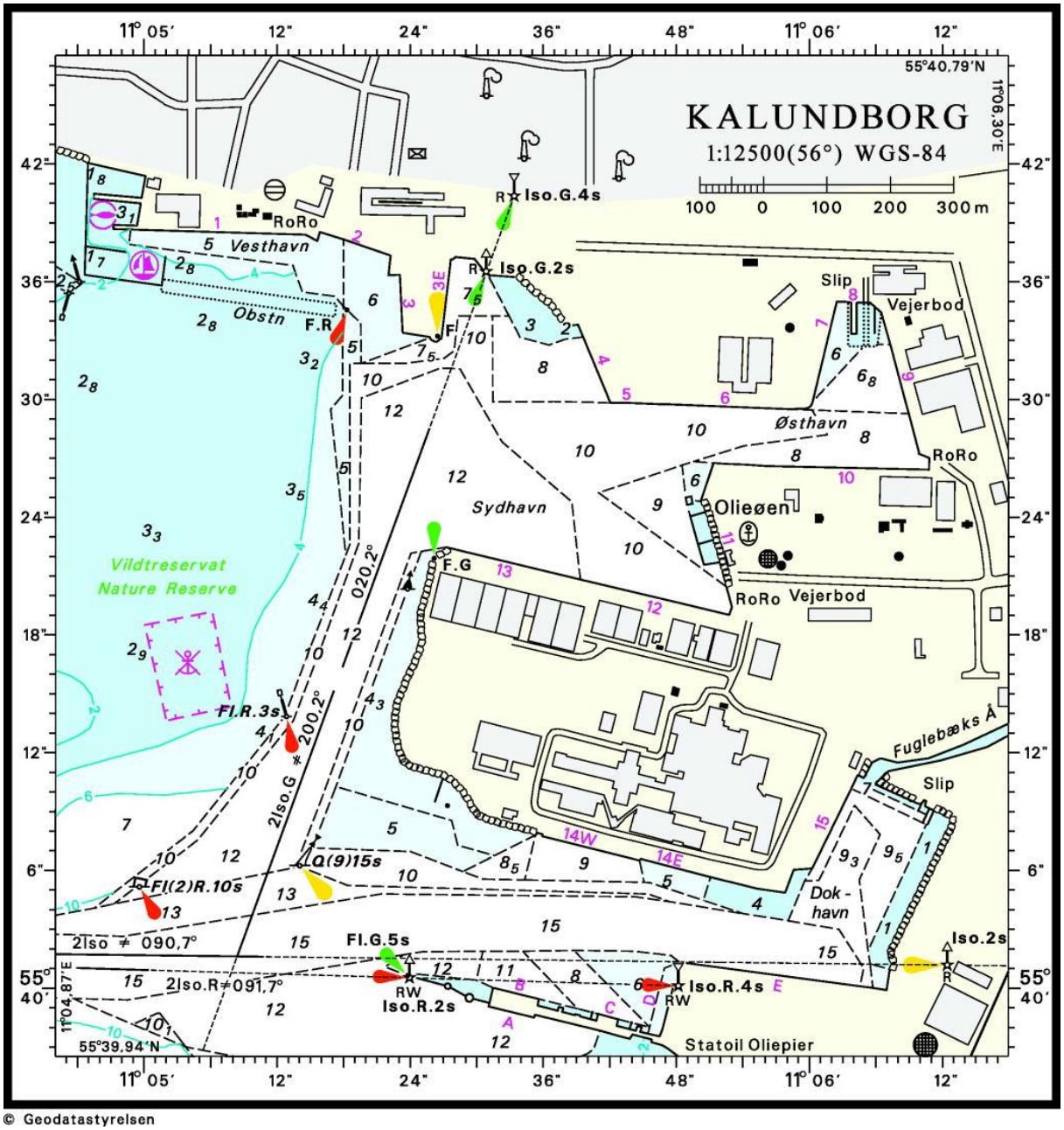
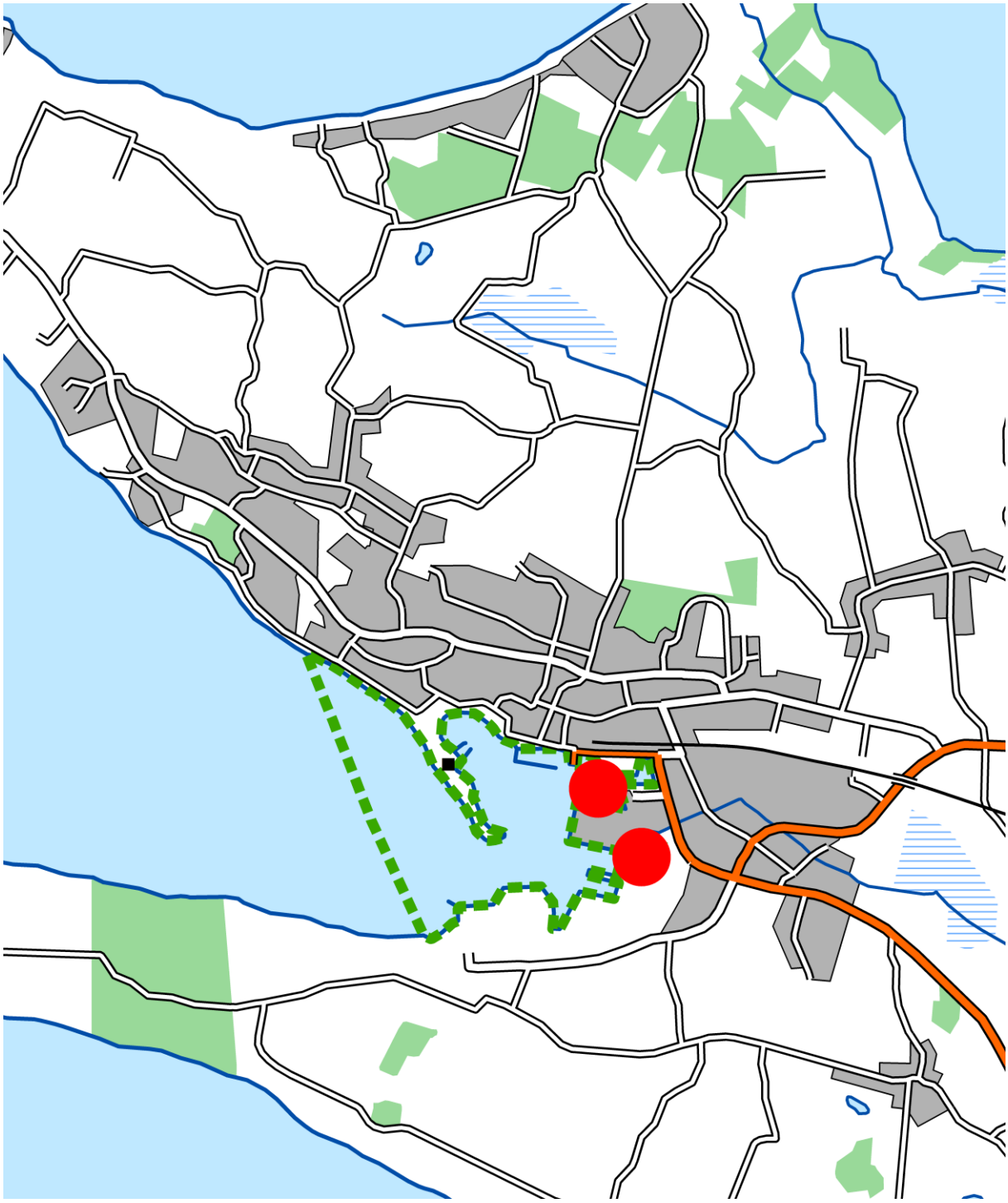
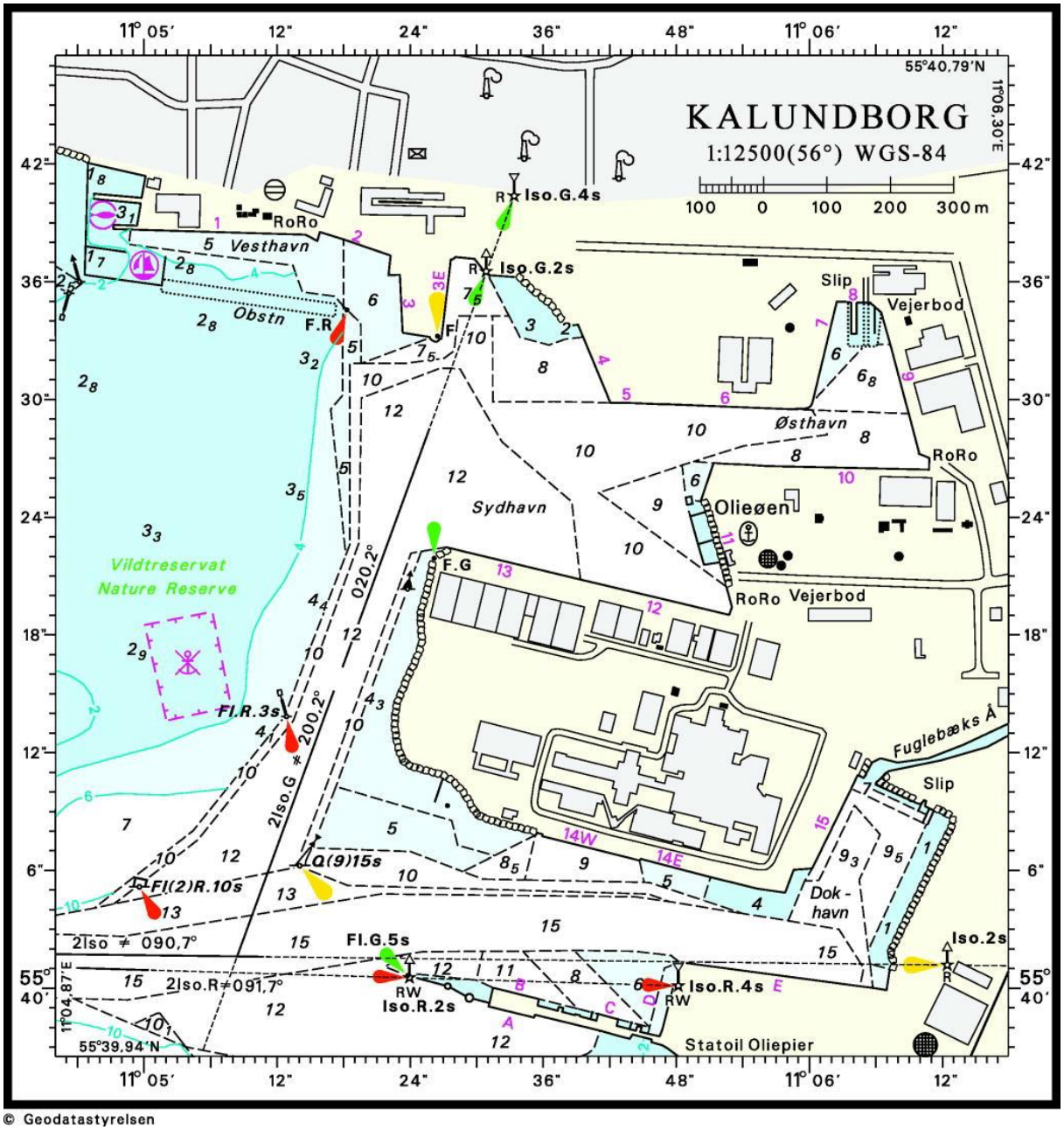
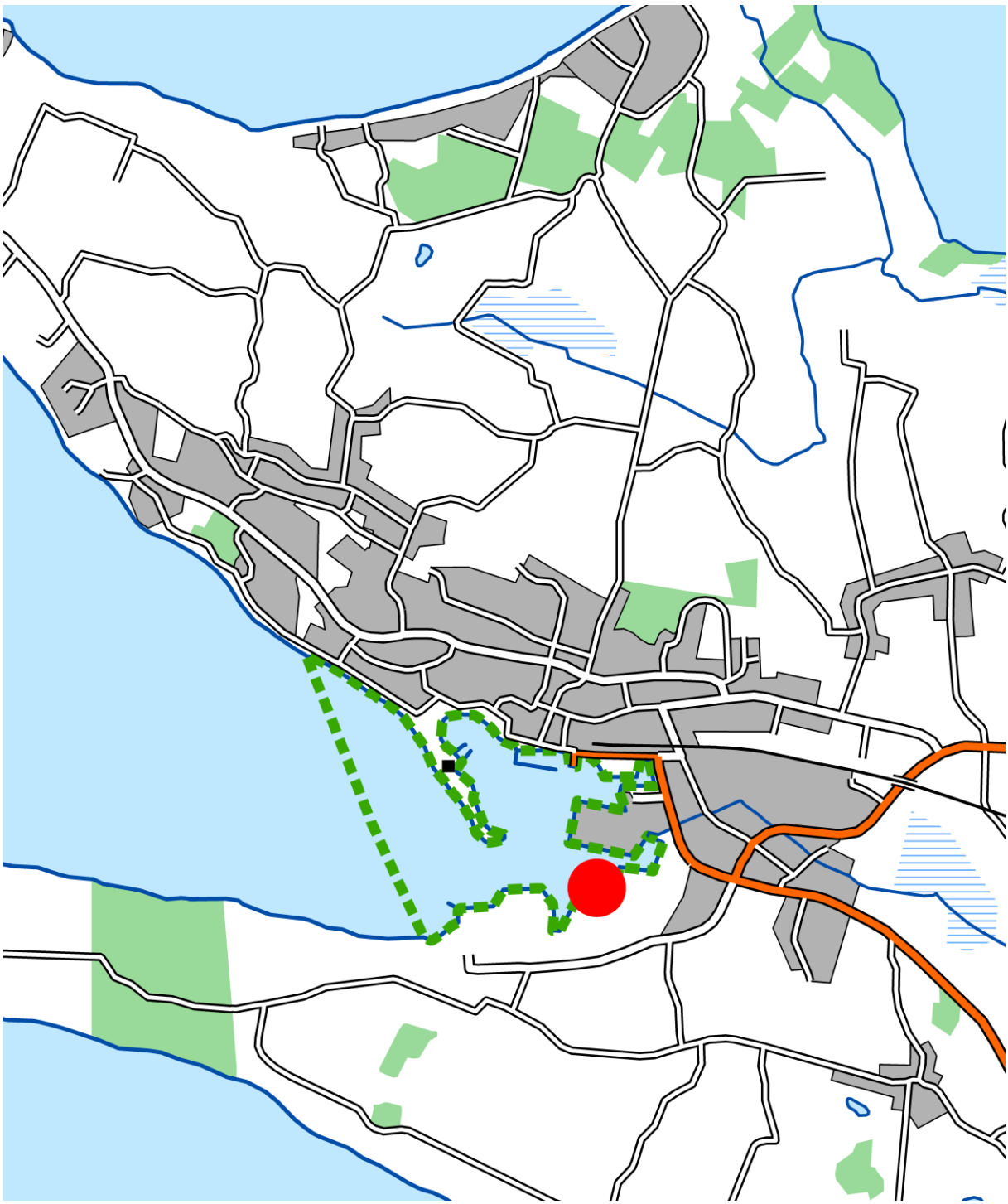


© Geodatastyrelsen

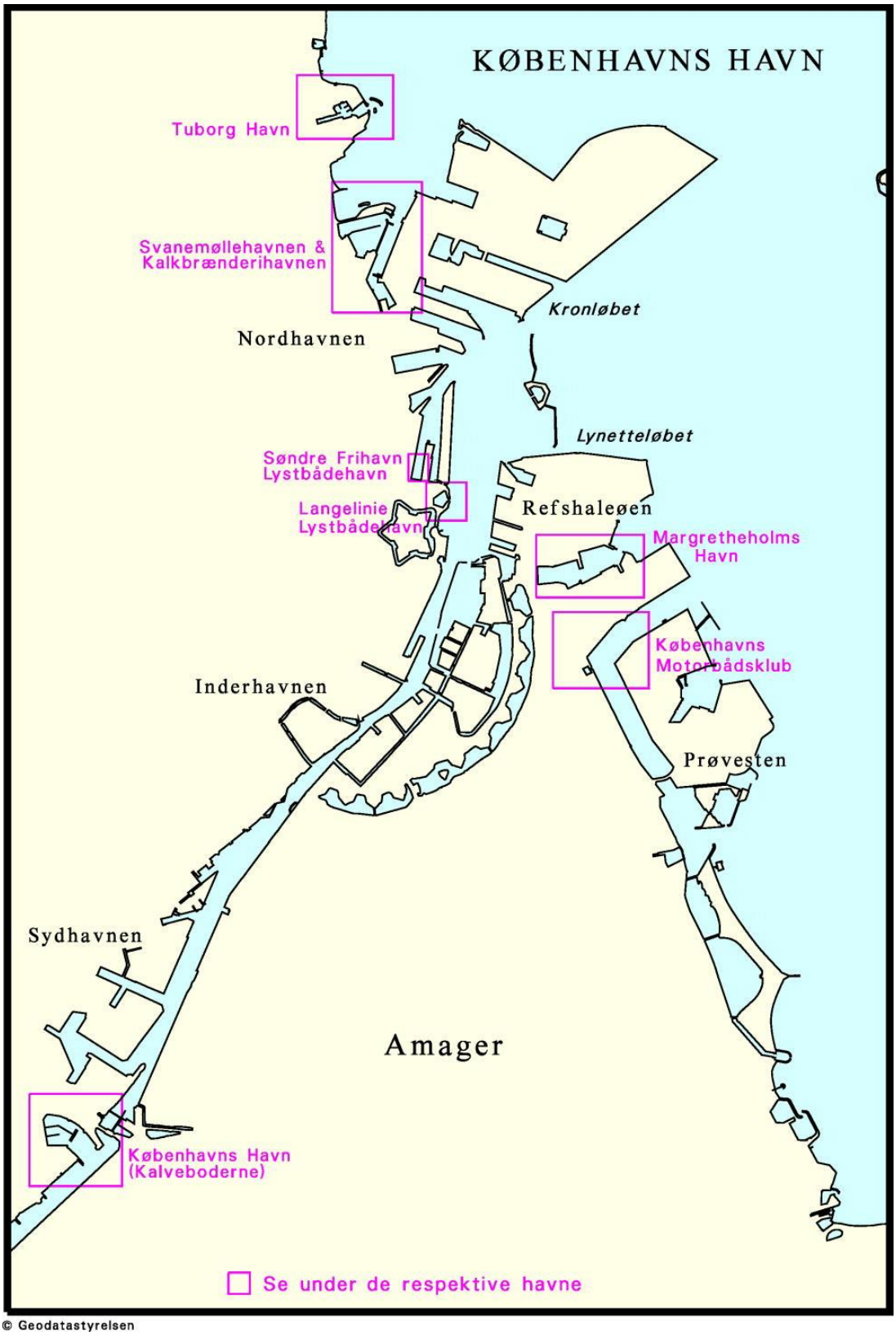


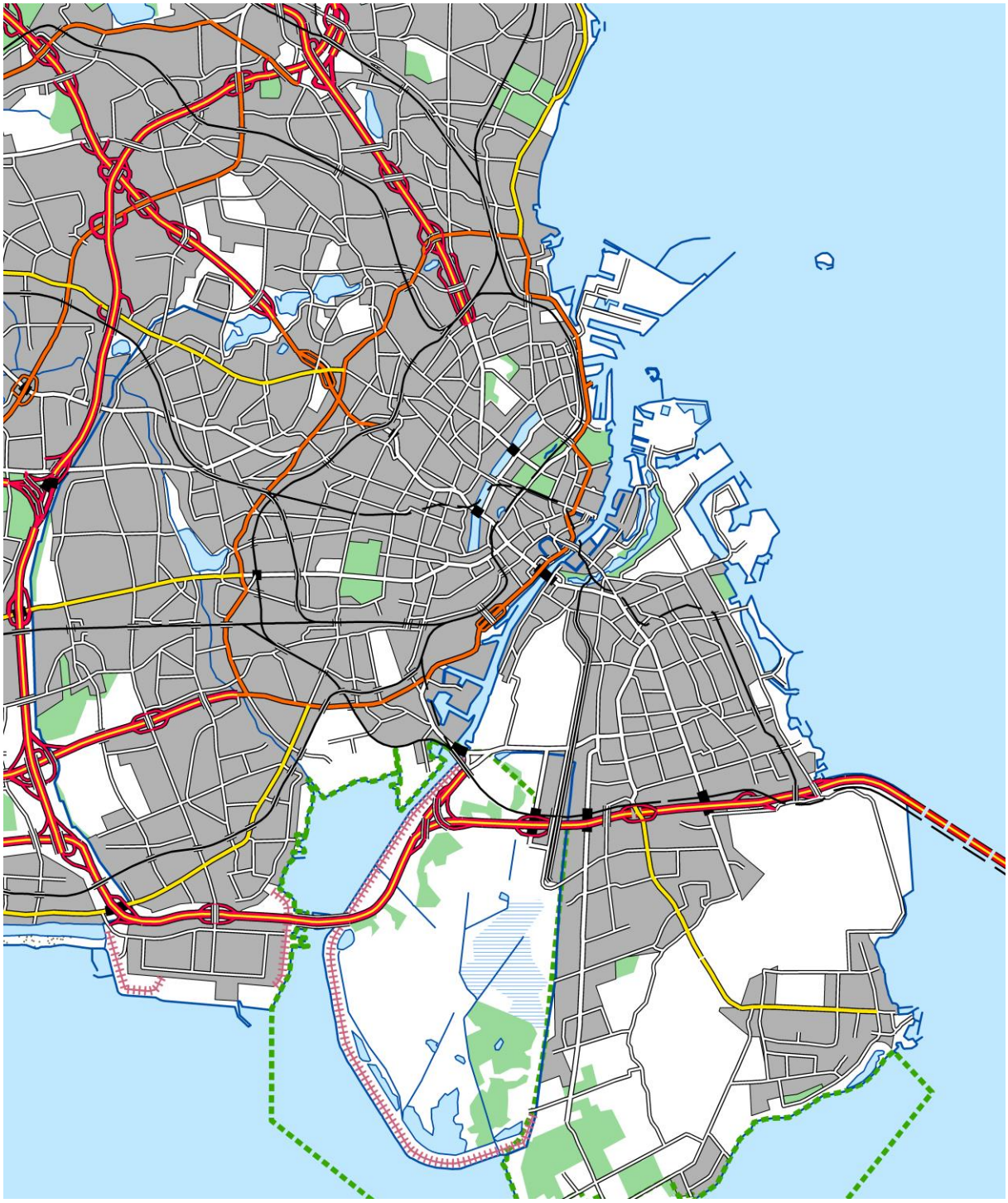
3.12 Kalundborg (Statoilhavnen)



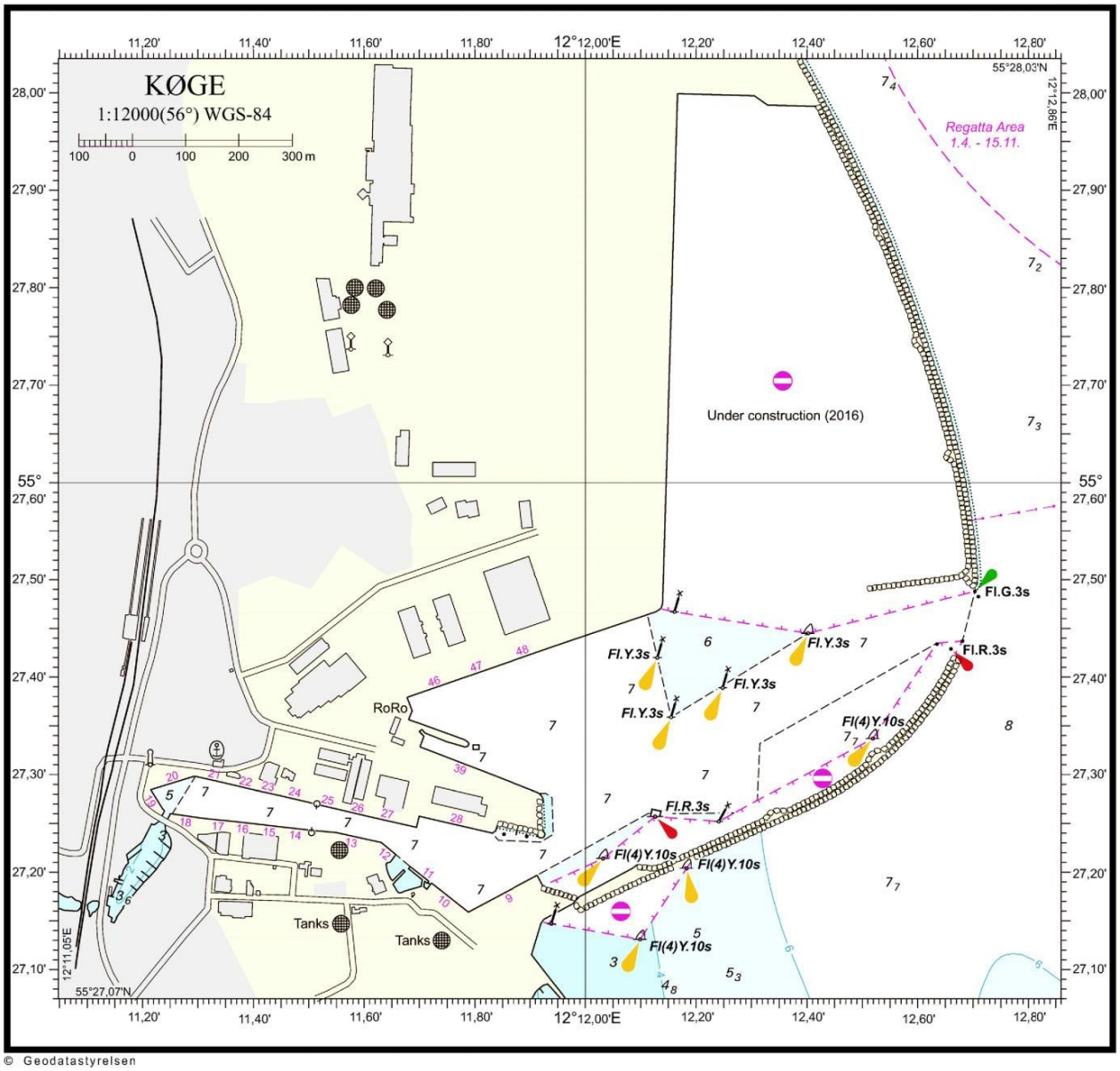


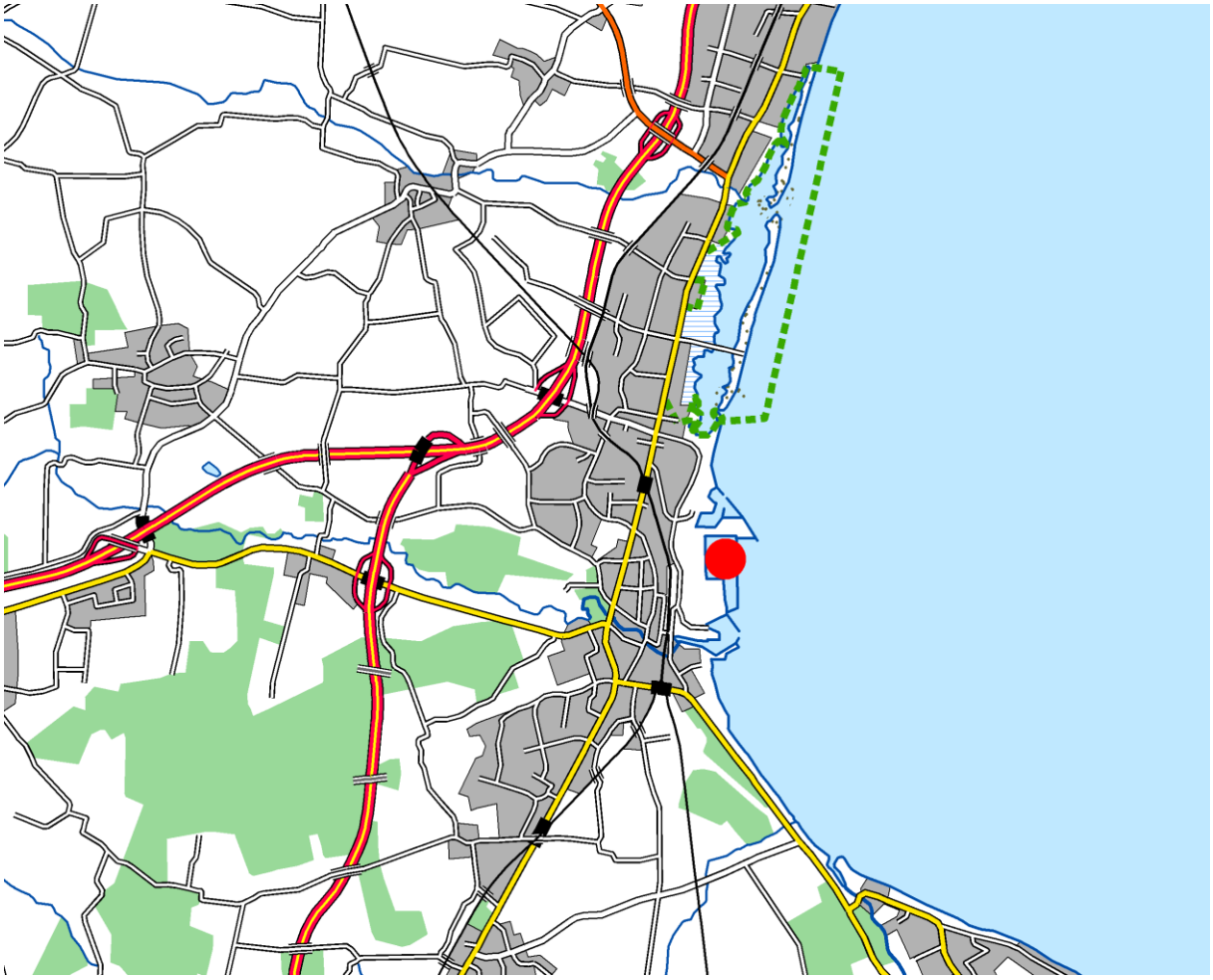
3.13 København



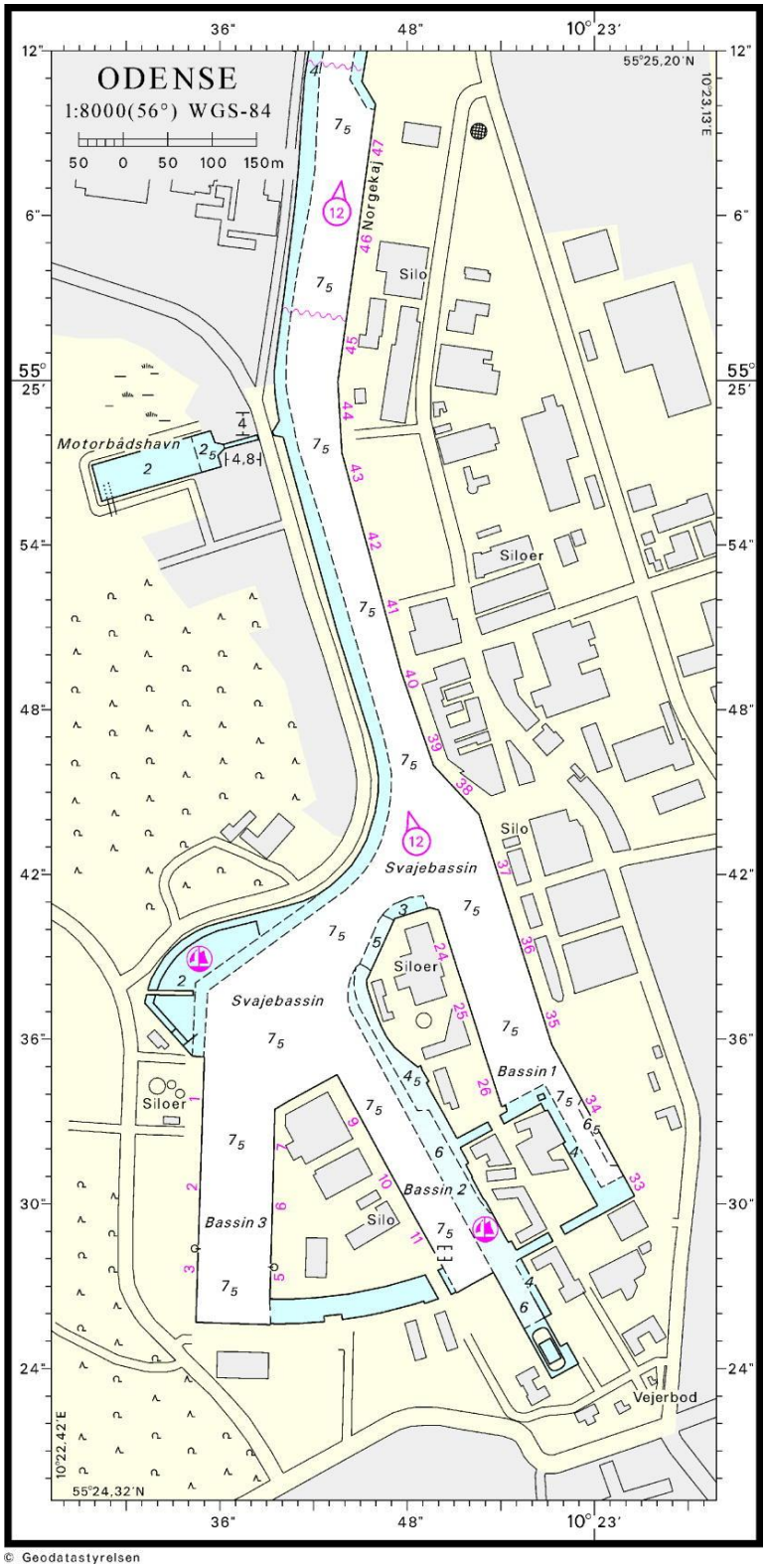


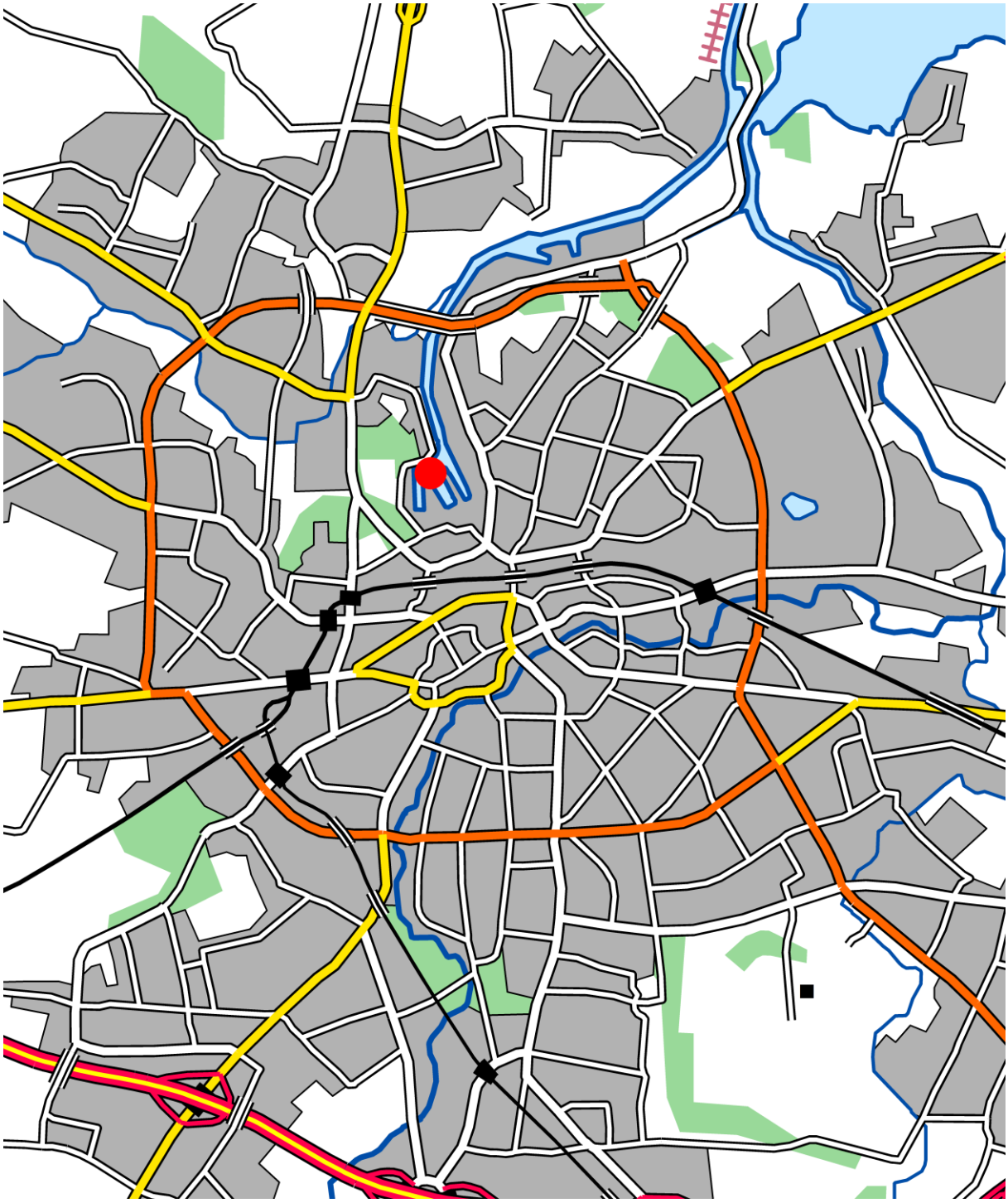
3.14 Køge


KØGE
1:12000(56°) WGS-84
100 0 100 200 300 m
Regatta Area
1.4. - 15.11.
Under construction (2016)
RoRo
Tanks
Tanks
Fl.G.3s
Fl.R.3s
Fl.R.3s
Fl.Y.3s
Fl.Y.3s
Fl.Y.3s
Fl.Y.3s
Fl(4)Y.10s
Fl(4)Y.10s
Fl(4)Y.10s
Fl(4)Y.10s
55°28,03'N
12°12,86'E
55°27,07'N
12°11,05'E
11,20' 11,40' 11,60' 11,80' 12°12,00'E 12,20' 12,40' 12,60' 12,80'
28,00' 27,90' 27,80' 27,70' 55° 27,60' 27,50' 27,40' 27,30' 27,20' 27,10'
© Geodatastyrelsen

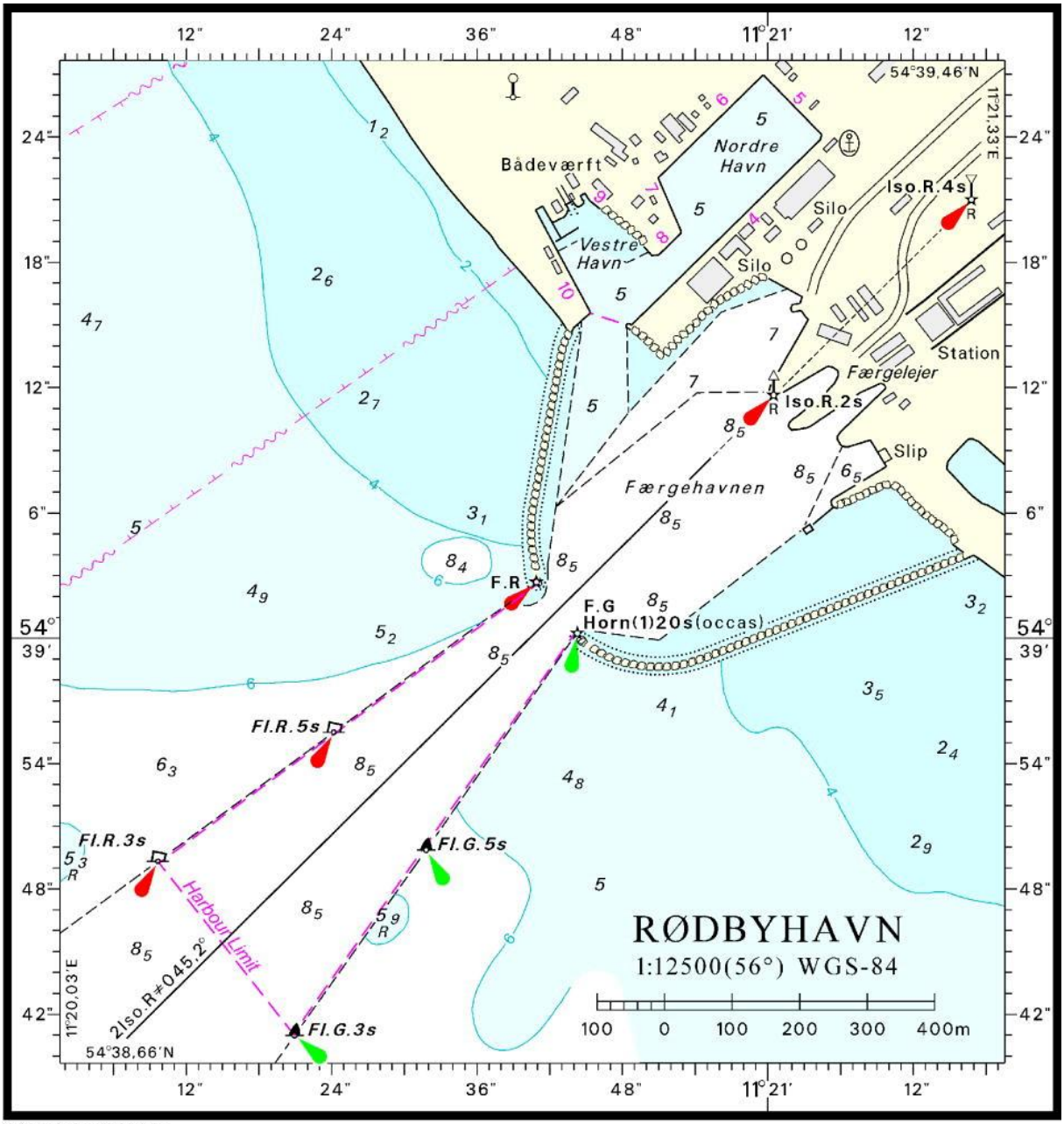


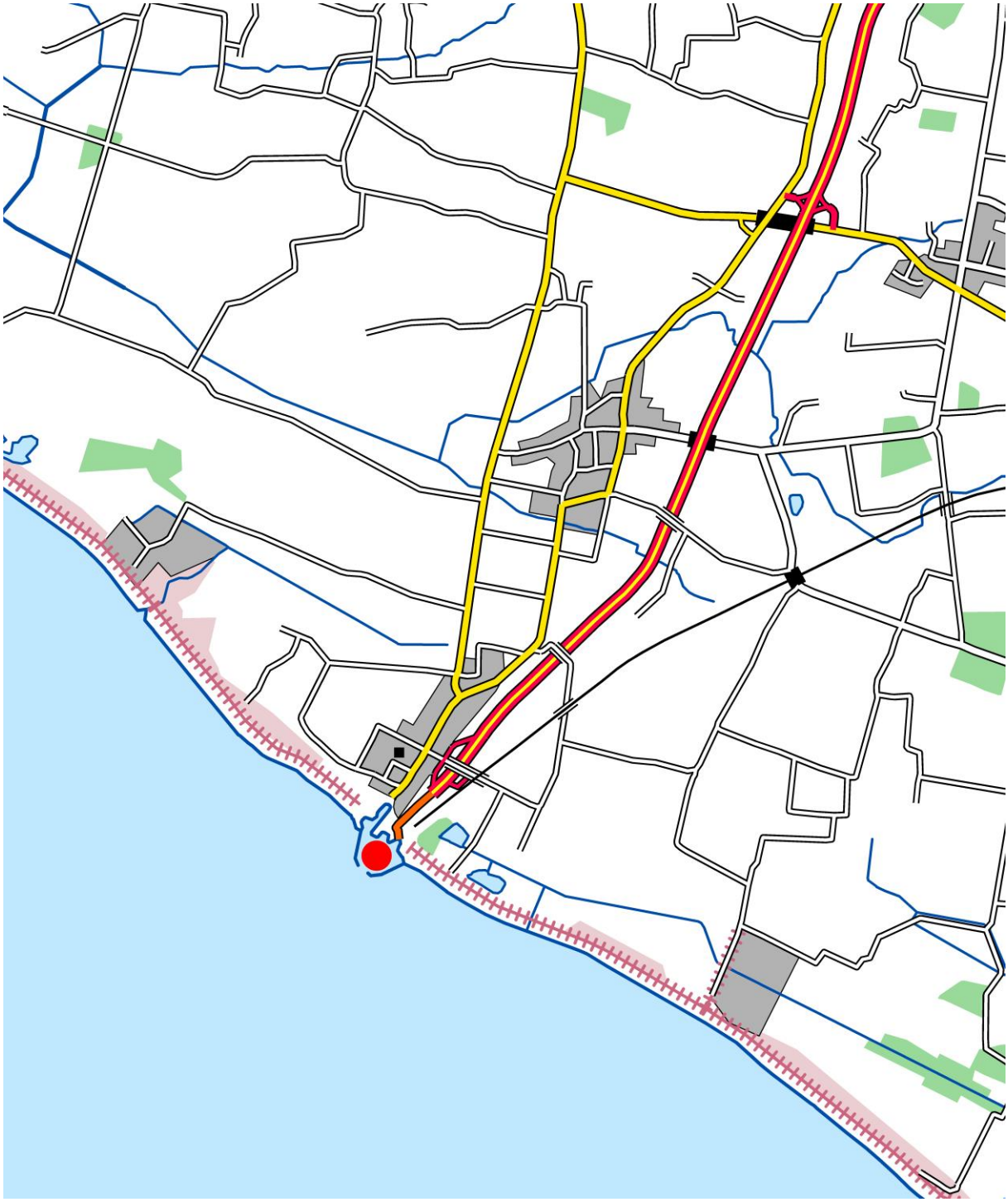
3.15 Odense


ODENSE
1:8000(56°) WGS-84
50 0 50 100 150m
36"
48"
10° 23'
55°25,20'N
10°23,13'E
Norgekaj
Silo
Motorbådshavn
4,8
Siloer
Svajebassin
Bassin 1
Bassin 2
Bassin 3
Vejerbod
10°22,42'E
55°24,32'N
© Geodatastyrelsen



3.16 Rødby





Rettigheder

NIVA Denmark is the name, water is our game

NIVA Denmark Water Research is a regional office of the Norwegian Institute for Water Research (NIVA) and has just recently been established to resolve environmental issues concerning the freshwater and marine systems that relate to Denmark.

NIVA Denmark has primary focus on research-based implementation of a number of EU's directives inter alia the Water Framework Directive and the Marine Strategy Framework Directive together with international conventions (HELCOM, OSPAR, BDC). We occasionally provide consultancy to authorities and small and medium-sized companies.

NIVA Denmark is a a place for practice, observation, testing and synthesis. Key research and test areas include eutrophication, hazardous substances, biodiversity, and ecosystem health as well as the implications of multiple human activities in marine waters and in streams, rivers and lakes. We develop indicators, monitoring methods and tools to assess the state of an ecosystem in order to carry out analyses and contribute to evidence based and sustainable solutions to the challenges we and the environment face.

NIVA Denmark, as a regional office to NIVA has thus the backing of more than 200 dedicated researchers and experts.

NIVA Denmark

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