

Another possible risk for the Mediterranean Sea? *Aspergillus sydowii* discovered in the Port of Genoa (Ligurian Sea, Italy)

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Abstract

Aspergillus sydowii is a cosmopolitan fungus that has been responsible for the mass destruction of coral in the Caribbean Sea over the last 15 years. To our knowledge, this study has found the first case of *A. sydowii* in the Mediterranean Sea, in marine-bottom sediments, water and calcareous shells of bivalve molluscs sampled during a campaign to characterise the mycobiota in the Port of Genoa (Italy). The area is characterised by adverse environmental conditions (high turbidity, organic pollution and high concentrations of phosphorus and nitrogen compounds). These parameters, in combination with a rising temperature, could contribute to *A. sydowii* bloom and dispersal. This fungal strain may have been imported into the Port of Genoa in the bilge water of vessels or by torrent input. This work represents the first step in the implementation of a monitoring programme to safeguard calcareous sponges and sea fan corals endemic in the Mediterranean Sea.

Aspergillus sydowii (Thom and Church) is a common, cosmopolitan, typically saprotrophic fungus that has been found in a variety of terrestrial environments, including deglaciated soils in Alaska, alpine habitats, and various soils in tropical regions, as well as in many different plant materials (Domsch et al., 2007; Klich, 2002). From a systematic point of view, *A. sydowii* belongs to the *Aspergillus* section *Versicolores* and is phenotypically closely related to *Aspergillus versicolor* (Jurjevic et al., 2012; Klich, 1993, 2002). Many *Aspergillus* species are opportunistic pathogens in stressed and immune-compromised hosts, including humans, fish, marine mammals, insects and plants (De Hoog et al., 2009; Zotti et al., 2015). Similarly, *A. sydowii* is able to cause diseases in plants (Munkvold, 2003) and animals (Pier and Richard, 1992), including humans (Nagarajan et al., 2014; Takahata et al., 2008). In marine environments, this pathogenic invader has been found in the West Indian Sea (Geiser et al., 1998), the Caribbean Sea (Alker et al., 2001) and, more recently, in Australian coastal waters (Hayashi et al., 2016). Starting from the 1980s (Guzmán and Cortés, 1984; Nagelkerken et al., 1997), this pathogenic invader has been implicated in the occurrence of aspergillosis in sea-fan corals (*Gorgonia ventalina* Linnaeus) with the consequent formation of tumours, galls, tissue necrosis leading to lesions, and purpling of degenerated tissue and the death of the corals (Dube et al., 2002). Some data indicate the presence of live *Aspergillus* spp. conidia in recent dust events (Garrison et al., 2003); hence, the hypothesis that *A. sydowii* has colonised marine ecosystems through wind dispersion, as in the Sahara-Sahel region of Africa (Weir-Brush et al., 2004). Confirming the role of winds in transporting fungi in the marine environment, Rypien (2008) reported that the powders could be transported through the Atlantic with prevailing winds and deposited in the Caribbean Sea, and Hayashi et al. (2016) reported an extensive *A. sydowii* marine fungal bloom after a dust storm event in Australia in 2009. Thus, one of the carriers of this coral illness could be atmospheric transport.

At present, little information is available on the environmental factors that can facilitate *A. sydowii* bloom in marine environment (Alker et al., 2001; Hallegraeff et al., 2014). The growth of pathogenic *A. sydowii* may be facilitated by environmental stress conditions (Garrison et al., 2003) such as organic pollution, lack of oxygen, or high levels of phosphorus and nitrogen (Burge et al., 2013; Ellner et al., 2007; Parekh and Chhatpar, 1986). Temperature also seems to be a crucial environmental factor for *A. sydowii* diffusion and pathogenicity, as indicated by Ward et al. (2007) who found that pathogen optima and host optima occurred in the same temperature ranges, thus giving the pathogen an opportunity to establish itself before host resistance is maximal.

This work is aimed at reporting the first survey of *A. sydowii* in the Mediterranean Sea: the fungus was found in the Port of Genoa (Liguria, north-western Italy; Fig. 1) in marine-bottom sediments, water and calcareous shells of bivalve molluscs (*Mytilus galloprovincialis*) during a campaign to characterise the fungal community of the port.

The Port of Genoa has a total area of $7 \cdot 10^6$ m² and 47 km of shipping lanes, and handled $2.2 \cdot 10^6$ Twenty-foot Equivalent Units of containers, $51.3 \cdot 10^6$ tons of goods, and 6000 vessel moorings in 2015.

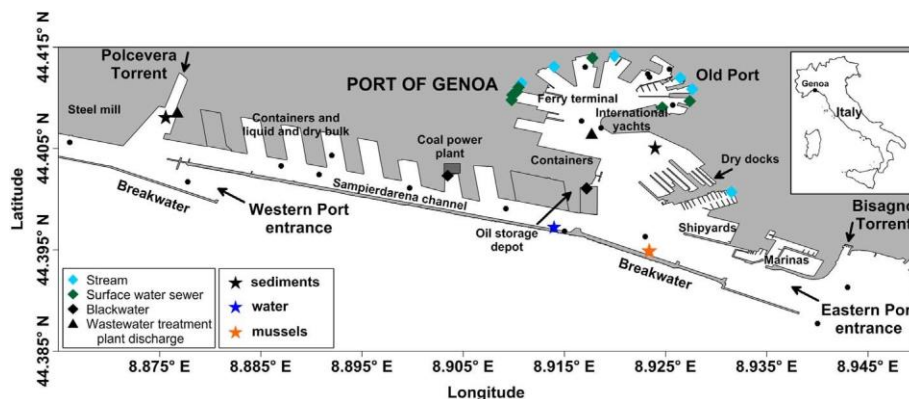


Fig. 1. Port of Genoa: localisation of the sampling stations (black points) and main features and activities of the port. Stars show the position of the samples characterised by the presence of the *Aspergillus sydowii*, and are coloured according to the studied matrix: black - bottom sediments; blue - surface water; orange - mussels. The different discharges inside the port are shown with rhombus and triangles. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Several commercial activities are present inside the port and include ferry and container terminals, dry docks, the coal power-plant of Genoa (ENEL, 2016), the port bulk terminal, shipyards, a steel mill (Mazzei et al., 2006), and different wastewater treatment plant discharges. On a daily basis, the Port of Genoa is subject to the traffic of ferries and cargo ships to and from other Italian and Mediterranean destinations (such as Tunis, Malta and Barcelona), and periodically, containerships to and from other Mediterranean countries (Morocco, Libya, Egypt, Lebanon, Spain), West Africa (Senegal, Ghana, Nigeria), North and South America (USA,

Brazil, Argentina), the Red Sea (Saudi Arabia), the Persian Gulf (United Arab Emirates), East and South-East Asia (South Korea, Singapore, Malaysia), and China (www.sech.it; www.messinaline.it; www.uasc.net/en; www.yml.com.tw).

The city of Genoa, with a land area of 243.6 km² and a population of 600,000, overlooks the port. The Genoa port basin (Fig. 1) includes the mouth of several urban streams and the mouth of the Bisagno and the Polcevera Torrents that have catchment surface areas of 93 km² and 140 km², respectively, and pass through small towns, quarries, factories and suburbs of the city of Genoa.

The principal winds that affect the Port of Genoa come from the NE (the most frequent wind) and the SE (Castino et al., 2003), and often, dust from the Sahara Desert is transported to Genoa during storms from the SE (Fiol et al., 2005; Israelevich et al., 2002; Ozer et al., 1998; Ozsoy et al., 2001). While the general sea circulation outside the port (Ligurian Sea) has a permanent cyclonic direction with meanders and eddies developing along the limits of the Gulf of Genoa, inside the port, the currents flow to the W along the Sampierdarena Channel when there is a NE wind, but the flow is more complicated in case of a SE wind (Capello et al., 2010; Cutroneo et al., 2012).

The temperature of the water inside the Port of Genoa ranges between 12 and 14 °C in winter and 14 and 27 °C in summer (with values reaching 29 °C in the surface layer of the innermost quays); the salinity ranges between 37 and 38 Practical Salinity Units (PSU) in summer and winter, and 36 and 37 PSU in early spring and autumn. The port waters are rich in nutrients, faecal coliform, chlorophyll-*a* and Polycyclic Aromatic Hydrocarbons (PAHs) due to the input of sewage and industrial discharges. Dissolved oxygen shows variable values in the different parts of the port, with the minimum of 40–50% in the innermost part of the Old Port and at the mouth of the Polcevera Torrent (Fig. 1). Generally, the turbidity ranges between 5 and 15 Formazin Turbidity Units, but it may undergo great changes as a result of heavy rain and ship manoeuvres causing the resuspension of the bottom sediments (Capello et al., 2015; Cutroneo et al., 2014; Ruggieri et al., 2011).

Samples were collected inside the Port of Genoa (Fig. 1) between December 2015 and January 2016 and consisted of bottom sediments collected with a Van Veen grab, surface water sampled with a Niskin bottle, and mussels taken from the internal side of the port breakwater. All the samples were stored in sterile plastic jars and ice boxes and immediately stored at 5 ± 1 °C in the laboratory to maintain their insitu chemical and physical characteristics and avoid the decomposition of the organic components. In this paper, only the samples where the *A. sydowii* was collected are considered (stars in Fig. 1).

Within 24 h of sampling, the isolation of fungal strains was performed using a different method for each substrate. From the bottom sediments, the fungal strains were isolated using a modified dilution plate technique (Cecchi et al., 2017a, 2017b; Zotti et al., 2014) which permits isolation of viable fungal strains. The dilutions were obtained by combining 1 g of marine sediment with 100 mL of sterile water in vials. Thereafter, a portion of the mixing obtained (1 mL) was inoculated in Petri dishes (diameter 9 cm) on two non-specific culture media prepared with autoclaved sea water: MEAs + C (Sea Malt Extract Agar with the addition of Chloramphenicol), and RBs (Sea Rose Bengal). Conversely, in order to isolate fungal strains in the calcareous shells of mussels, the shells were scratched and washed with sterile sea water; 3 mL of washing water were homogeneously scraped onto Petri dishes (diameter 9 cm) containing culture medium RBs. Instead, in order to isolate fungal strains in the seawater, 1 mL of the sea water was homogeneously spread on the surface of culture media MEAs + C. All the inoculated Petri dishes were incubated in the dark at 24 ± 1 °C for 2–3 weeks to allow the optimal growth of the fungal strains. Later, all the vital fungal strains were isolated in axenic cultures using test tubes containing MEA.

The fungal strains were deposited at the Mycological Laboratory of the Department of Earth, Environment and Life Sciences of the University of Genoa.

The strains were identified with a polybasic approach. At first, the micro- and macromorphological characteristics were evaluated with the help of specific papers or taxonomical keys (Klich, 1993, 2002; Raper and Fennel, 1977; Samson et al., 2014; Siqueira et al., 2016). For this purpose, an optical microscope was employed. Then, a molecular technique was adopted which involved genomic DNA extraction, PCR amplification and DNA sequencing. The genomic DNA was extracted from 100 mg of fresh fungal culture using a modified CTAB method (Doyle, 1987). The morphological identifications were confirmed by amplification of the β -tubulin gene using the primers Bt2a and Bt2b (Glass and Donaldson, 1995) and the ITS region amplification using the universal primers ITS1F/ITS4 (Gardes and Bruns, 1993). The PCR products were purified and sequenced by Macrogen Inc. (Seoul, Republic of Korea). The sequence assembly and editing were performed with the sequence analysis software Sequencher® (Gene Codes Corporation, ver. 5.2). The sequences obtained were compared with the Gen Bank database using the BLASTN algorithm.

Isolates representing *A. sydowii* were found in four samples (two sediment samples, one water sample and one mussel sample) distributed throughout the Port of Genoa (Fig. 1). Pure cultures were obtained by repetitive culturing of the fungal strains in test tubes, starting from the inoculation of the samples.

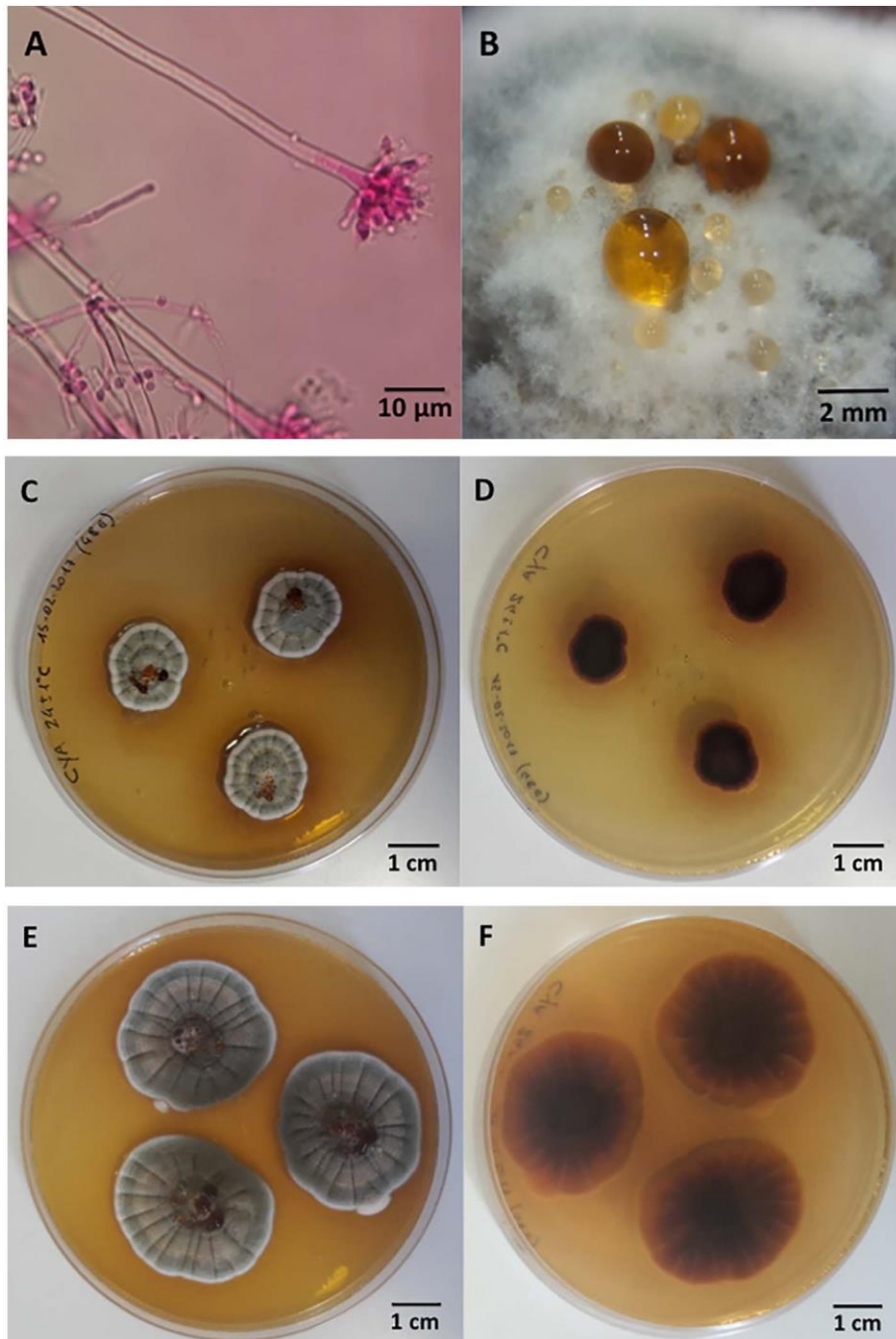


Fig. 2. A: biseriatic conidial head under optical microscope (40×/0.75). B: detail of the yellowish to reddish-brown exudate under stereomicroscope. C: plate of *A. sydowii* on CYA after 7 days. D: reverse plate of *A. sydowii* on CYA after 7 days. E: plate of *A. sydowii* on CYA after 14 days. F: reverse plate of *A. sydowii* on CYA after 14 days.

from very similar species of *A. versicolor*; in fact, in particular, in *A. sydowii* the colonies appear as a greyish-turquoise colour, instead of green, on CYA25 (24C-E 3-5; Kornerup and Wanscher, 1967) and relatively small in diameter (25 mm in 7 days). The exudate is clear to yellowish to reddish brown (8D-E 5-7; Kornerup and Wanscher, 1967); a reddish-brown soluble pigment is present and the reverse is brown (5C-E 6-7; Kornerup and Wanscher, 1967). The rough conidia are borne by small biseriatic conidial heads (10 μm) with uncoloured stipes (Fig. 2A).

The presence of *A. sydowii* in the Port of Genoa may be due to the transport of the Sahara Desert dust during SE storms (Ozer et al., 1998), such as in the case of the Caribbean Sea (Garrison et al., 2003; Hallegraeff et al., 2014), or the transport in the bilge water of the container vessels travelling on the Asia-Genoa or Africa-Genoa routes. Another source of the *A. sydowii* inside the

Port of Genoa could be a general terrestrial runoff (Smith et al., 2013) and the input from the Polcevera and Bisagno Torrents, but there are no reports of the presence *A. sydowii* in Liguria that can support this hypothesis; a recent terrestrial record of *A. sydowii* in Italy was only from living bats (*Hypsugo savii* Bonaparte) in Turin, north-western Italy (Voyron et al., 2011).

Of course, the limited presence of *A. sydowii* could be due to the scarce fungal sampling in the Ligurian Sea and the relatively low water temperature in winter which can inhibit the development of *A. sydowii*. Moreover, the absence of sea fans, such as *G. ventalina*, inside the Port of Genoa does not help the development of this opportunistic pathogen. In fact, only *Leptogorgia sarmentosa* Esper is present in the Port of Genoa, but this species has very subtle ramifications with specimens often isolated and, therefore, it would be difficult to attack.

However, some researchers reported that *A. sydowii* was isolated not only from calcareous sea fans but also from healthy calcareous marine sponges (Ein-Gil et al., 2009). In fact, some fungi are known to bore into carbonate substrates where they utilise organic substances incorporated in mineralised tissues (Bak and Laane, 1987; Che et al., 1996). Hence, it seems plausible that *A. sydowii* prefers carbonate substrates in order to find a more stable micro-environment for its growth and propagation. This fact suggests that *A. sydowii* could spread from the Port of Genoa to other zones in the Ligurian and Mediterranean seas due to the considerable traffic of vessels, and could be transported by sea currents to more suitable environments and hosts.

Hence, acquiring more data on the interactions between *A. sydowii* and environmental factors is needed. This includes an evaluation of adverse environmental conditions and specific parameters, such as pollutants, temperature (Rosenberg and Ben Haim, 2002; Ward et al., 2007; Burge et al., 2013), nutrients (Bruno et al., 2001; Baker et al., 2007), water circulation (Dube et al., 2002), and growth substrates that may determine new sicknesses or increasing and spreading aspergillosis of sea-fan corals or sponges. Active management of these external stressors, especially nutrient inputs, sedimentation, and climate control are the only logical solutions to mitigate the effects of this coral disease (Rypien, 2008).

The present paper may represent the first step for the implementation of a monitoring study, which can help safeguard the calcareous sponges and sea fan corals endemic in the Ligurian and Mediterranean seas (e.g. within the Protected Marine Areas of Portofino and Bergeggi, 20 and 40–km distant from the Port of Genoa, respectively). The research in this field will continue to determine the microbiome of healthy *G. ventalina* vs. infected ones in the same region to observe possible changes in the “nature” of the fungus from commensal to opportunistic pathogen. In addition, we will check the pathogenicity of the isolated strains following several approaches. The idea is to use Koch's postulates and population genetic methods to identify possible patterns of genetic diversity and relationships between environmental and disease-causing strains of this fungus. Eventually, other studies will be in order to define whether *A. sydowii* may also be considered a preferential pathogen for *G. ventalina* or may attack other sea fan species also present in the Ligurian and Mediterranean seas, such as *Eunicella verrucosa* Pallas, *Eunicella cavolini* Koch or *L. sarmentosa*.

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