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No more lake balls (*Aegagropila linnaei* Kützing, Cladophorophyceae, Chlorophyta) in The Netherlands?

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Abstract *Aegagropila linnaei*, a freshwater green macroalga, had been abundant in several locations in The Netherlands before the 1960s. Both the 'lake ball' form of this alga and dense unattached mats floating over the sediment have been described from these locations. After 1967, this species has not been recorded anymore from The Netherlands. In 2007, several historical collection sites were surveyed for extant populations of *A. linnaei*. All habitats have changed drastically during the last 50 years and were affected severely by eutrophication. Populations of *A. linnaei* seem to have become extinct in all but one location (Boven Wijde, province Overijssel), where we found very small amounts of attached filaments. The attached form had not been reported previously from The Netherlands. Environmental conditions do not seem suitable anymore to maintain extensive unattached growth forms including the enigmatic lake balls, and the species must be regarded as threatened in The Netherlands and we propose to include *A. linnaei* in a national red list. The decline of populations elsewhere is reviewed and discussed in this paper. In addition to morphological identification of the attached filaments, partial sequences of the nuclear large subunit rDNA were generated and compared with different growth forms and habitats from several other locations outside The Netherlands. The sequences confirm the identity of the Dutch material and indicate very little divergence both between populations in different locations and between different growth forms.

Keywords *Cladophora aegagropila* · Eutrophication · Threatened freshwater algae · Lake balls · LSU rDNA · Marimo · Molecular sequences · Red list

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Introduction

The green macroalga *Aegagropila linnaei* Kützing (Cladophorophyceae) occurs in freshwater and some brackish environments and has essentially a palaearctic distribution (van den Hoek 1963; Pankow 1965). The simple morphology consists of stiff, branched uniseriate filaments and is very similar to members of the related genus *Cladophora* Kützing. *A. linnaei* is morphologically distinct from *Cladophora* spp. by the lateral or subterminal, often opposite, sometimes serial insertion of branches; irregular cell shape and unrestricted insertion positions of branches especially in the basal parts; and development of rhizoids from the base of cells which can produce terminal haptera (van den Hoek 1963; Leliaert and Boedeker 2007; Soejima et al. 2008).

The species is presumed to reproduce mainly or entirely vegetatively by means of fragmentation (Brand 1902; Acton 1916; van den Hoek 1963). Based on morphological similarities, the species was placed in the genus *Cladophora* (as *C. aegagropila* (L.) Rabenhorst, van den Hoek 1963), but has recently been recognized as its own monotypic genus based on molecular evidence. It is placed in a separate clade that is sister to the orders Siphonocladales and Cladophorales, together with several other species-poor, predominantly freshwater genera such as *Arnoldiella*, *Basicladia*, *Pithophora* and *Wittrockiella* (Hanyuda et al. 2002; Leliaert et al. 2003).

Aegagropila linnaei can occur in several different growth forms, depending on environmental conditions (Waern 1952; Sakai and Enomoto 1960; Niiyama 1989). The species is best known for the formation of spherical balls several centimeters in diameter, the so-called lake balls, marimo (in Japan), or moss balls (in aquarium shops). These balls consist of many interwoven filaments. In Japan, the lake balls are very popular and have been designated a 'special natural monument' (Kurogi 1980). The ball forms have also become very popular in the aquarium trade.

The species can grow as unattached mats, floating above the substrate in shallow water, or as attached epilithic or epizoic filaments. Different theories about the formation of the ball form have been proposed, but it is generally assumed that the formation is a mixture of mechanic processes through water motion and features intrinsic to the species that help entanglement, such as the stiff texture, the formation of rhizoids, and the growth pattern following the abrasion of apical cells while rolling on the sediment (Wesenberg-Lund 1903; Acton 1916; van den Hoek 1963; Kurogi 1980; Niiyama 1989; Einarsson et al. 2004). It has been shown in culture experiments that stable balls can be produced when exposing loose filaments to a rolling motion (Nakazawa 1973).

A recent study from Japan also contributing to the conservation of *A. linnaei* and the management of its habitats indicates the possibility of genetic differentiation between different growth forms, and it emphasizes the necessity to extend conservation efforts to epilithic populations as well (Soejima et al. 2008).

Floating balls or unattached mats of *A. linnaei* have been found in several locations in The Netherlands: in Lake Naardermeer in North Holland (Koster 1959), in the lake system Loosdrechtse Plassen in North Holland (Koster 1959), in Lake Zwarte Broek in Friesland (Kops et al. 1911; Koster 1959), and in several parts of the lake system 'De Wieden' in the province Overijssel (Koster 1959; Segal and Groenhart 1967). Since then, *Aegagropila* balls or mats have not been reported again, so the last record of this species in The Netherlands is more than 40 years old. Attached forms had never been reported from The Netherlands.

Taking into account the long period during which *A. linnaei* had not been recorded and the changes in the environment in the meantime, it was assumed that this species had disappeared from its original habitats. Therefore, the historical collection sites were visited to check whether this species and its characteristic ball-shaped growth form still might exist in The Netherlands. We further discuss the situation and threat to this species elsewhere. Partial LSU rDNA sequences were generated to verify morphological identifications of putative material of *A. linnaei* and to investigate possible genetic divergence between different growth forms, as well as between populations from different habitats and locations.

Materials and methods

Herbarium and literature survey of Dutch locations

During a survey on the global distribution of *A. linnaei* based on herbarium specimens, it became obvious that there are very few recent collections of this species in general, with the majority of the herbarium specimens being more than 100 years old. For findings from The Netherlands that are neither mentioned in available literature nor deposited in the collections of herbaria, we contacted several Dutch organizations that carry out monitoring of aquatic vegetation and/or water quality in different areas of The Netherlands. These include Stichting Natuurmonumenten, Stichting Floron, Landelijk Informatiecentrum Kranswieren (LIK), Waternet, Waterschap Reest and Wieden, and Wetterskip Fryslân. Also several boat rental businesses and dive clubs in The Netherlands were contacted for possible amateur sightings of 'lake balls'. The locations in the resulting

Table 1 Locations in The Netherlands where *Aegagropila linnaei* has been reported from, with information on reported growth forms and abundance of *A. linnaei*, the date of the last record, and sources

Location (province)	Lat.	Long.	Depth (m)	Reported growth forms of *A. linnaei*	Notes on abundance	Last record	Herbarium collections[a]	Additional references
1. Naardermeer (North Holland)	N52°17′60″	E05°07′12″	1	Unattached mats, balls	Very abundant	1962	L, LD	Koster (1959)
2. Wijde Blik (North Holland)	N52°13′12″	E05°02′60″	1–2	Ball		1955	L	
3. De Wieden (Overijssel)								
Beulaker Wijde	N52°42′03″	E06°03′36″	1	Ball		1938		Koster (1959)
Boven Wijde	N52°43′48″	E06°06′36″	1	Tufts (herbarium), ball (literature)		1944	L	
Duinigermeer	N52°43′12″	E06°00′00″	1	Ball		1967		Segal and Groenhart (1967)
Zuideindigerwiede	N52°42′36″	E06°04′48″	1	Balls		1951	L	Segal and Groenhart (1967)
Molengat	N52°44′24″	E6°05′24″	1–(3)	Unattached mats	Numerous; dense masses	1960	L, LD	Segal and Groenhart (1967), van den Hoek (1963)
4. Zwarte Broek and nearby ditches (Friesland)	N53°15′00″	E05°56′24″	1	Unattached tufts, balls	Not common, but numerous	1906	BI, BR, BRNU, L, NY, PC	Kops et al. (1911)

The location numbers correspond to Fig. 1

[a] Herbarium acronyms follow Holmgren et al. (1990)

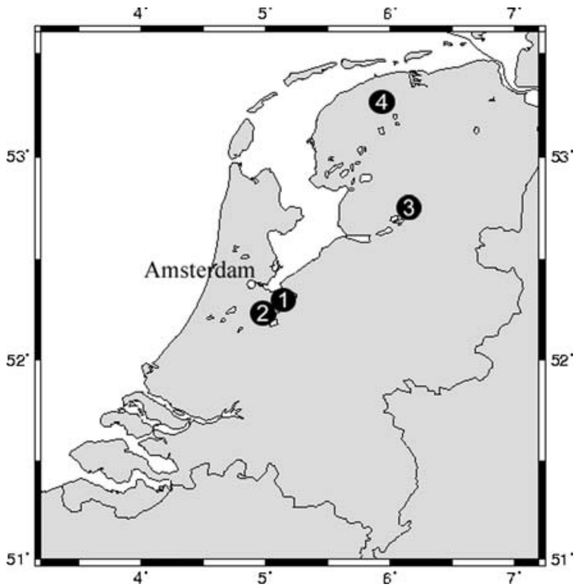

Fig. 1 Map of The Netherlands indicating the historical collection sites of *Aegagropila linnaei* by numbers. *1* Naardermeer, *2* Loosdrechtse Plassen, *3* De Wieden area, *4* Zwarte Broek

list (Table 1) were the candidates for field work to search for extant populations in The Netherlands.

Field work

We visited six locations during seven field trips, covering most places where *A. linnaei* had been found previously (Fig. 1), plus one additional location that was pointed out by amateur divers ('De Groene Heuvels'). The Beulaker Wijde, the Duinigermeer, and the Zuideindigerwiede could not be visited due to time constraints. Information about the locations and employed field methods is summarized in Table 2. Field methods depended on local conditions, i.e., size of the lake, shore accessibility, underwater visibility, and availability of a boat. Wherever information on the precise location of previous collections was available, field work was focused in those areas of the lakes (i.e., the Mennegat in the Naardermeer and the A. Lambertskade in the Loosdrechtse Plassen). If the underwater visibility was too poor for snorkeling or SCUBA diving, the sampling consisted of dredging with a standard garden rake using a boat or wading in shallow water, and shore observations. Special attention was paid to submerged solid substrates, i.e., mussel shells and stones. Shore observations were concentrated to areas with reed stands and leeward bays or shores. Material resembling *A. linnaei* was brought back to the laboratory and examined under a microscope. Verified material of *A. linnaei* was vouchered and stored in L (the National Herbarium of The Netherlands, Leiden branch; see Table 3 for details).

DNA sequence analysis

Partial large subunit (LSU) rDNA sequences from six specimens of *A. linnaei* were analyzed to check the morphological identifications, covering different growth forms, habitats, and locations (including one Dutch site). Sample and collection information, voucher specimens, and GenBank accession numbers are given in Table 3. DNA was extracted from fresh material or specimens that had been desiccated in silica gel after collection (Chase and Hills 1991). Fresh material was processed as herbarium vouchers now deposited in L, plus additional liquid preservation in formaldehyde solution or ethanol. Total genomic DNA was isolated using the Chelex method (Goff and Moon 1993).

PCR amplifications were performed in a Biomed thermocycler with an initial denaturation step of 94°C for 5 min followed by 31 cycles of 30 s at 94°C, 30 s at 57°C, and 30 s at 72°C, with a final extension step of 5 min at 72°C. The reaction volume was 25 µl and consisted of 0.1–0.4 µg genomic DNA, 1.25 nmol of each dNTP, 6 pmol of each primer, 2.5 µl of 10× reaction buffer containing 1.5 mM $MgCl_2$ (Qiagen), 1 µl BSA (2.5%), 17.7 µl H_2O, and one unit of *Taq* polymerase (Qiagen). The first ~590 nucleotides of the LSU rDNA were amplified using the universal primers C′1 forward (5′-ACCCGCTGAATTTAAGCATAT-3′) and D2 reverse (5′-TCCGTGTTTCAAGACGG-3′; Hassouna et al. 1984; Leliaert et al. 2003). Amplifications were checked for correct size by electrophoresis on 1% agarose gels and subsequent staining with ethidium bromide. PCR products were purified with Montage PCR filter units (Millipore) or with ExoSAP-IT (USB Corporation) following the manufacturers' protocols. Cleaned PCR products were sent to Macrogen, South Korea, for sequencing. The final consensus sequences were constructed with Sequencher 4.0.5 software (GeneCodes), subsequently aligned by eye in Se-Al v2.0a11 (Rambaut 2007) and submitted to GenBank (see Table 3).

Table 2 Locations in The Netherlands that were visited in search of extant populations of *Aegagropila linnaei*, with information on fieldwork details, visibility, sediment, and observed vegetation

Location (province)	Field dates	Field methods	Visibility (m)	Sediment	Observations
1. Naardermeer (North Holland)	July 2007	Snorkeling	1	Sand	Dense vegetation of Characeae, numerous ball-shaped colonies of *Nostoc pruniforme*.
2. Wijde Blik (North Holland)	May 2007	Snorkeling, shore survey	0.5–1	Sand, mud in sheltered areas	No floating algae; barren sediment. Only macroalgae observed: *Cladophora glomerata*, *Gongrosira* sp. (on *Dreissena polymorpha*), *Vaucheria* sp.
3. De Wieden (Overijssel)					
Boven Wijde	June 2007	Snorkeling, shore survey	0.5	Mainly mud, sand on eastern shore	Floating on surface: *Cladophora glomerata*, *Oedogonium* sp., *Spirogyra sp*. On reed: *Chaetophora elegans*, *Rhizoclonium* sp. On *Anodonta anatina*: *Gongrosira* sp., one single tuft of *Aegagropila linnaei*
Molengat	June 2007	Raking, shore survey (boat)	<0.5	Mainly mud, sand on eastern shore	No floating algae. On sand: *Vaucheria* sp. Attached on wood and waterplants: *Cladophora glomerata*, *Cladophora fracta*, *Oedogonium* sp. *Anodonta anatina* and *Dreissena polymorpha* without epiphytic growth
	Nov 2007	SCUBA diving	<0.5		
4. Zwarte Broek (Friesland)	Aug 2007	Raking, shore survey	<0.1	Thick layer of mud	Floating on surface: *Cladophora glomerata*, *Hydrodictyon reticulatum*, *Lemna minor*, *Spirogyra* sp. *Anodonta* sp. without epiphytic growth

The location numbers correspond to Fig. 1

Results

Herbarium and literature survey of Dutch locations

In total, 27 herbarium specimens of *A. linnaei* from Dutch locations could be traced. The collection in L (see Holmgren et al. 1990 for herbarium abbreviations) contains the majority of the material (11 specimens) and covers six locations in The Netherlands: Boven Wijde, Molengat, Zuideindigerwiede (all in an area called 'De Wieden', province Overijssel), Mennegat (Naardermeer, province North Holland), Wijde Blik (Loosdrechtse Plassen, province North Holland), and Zwarte Broek (near Roodkerk, province Friesland; Fig. 1). Additional specimens from the same locations of *A. linnaei* were housed in nine other herbaria (BM, BR, BRNU, LD, M, NY, PC, UBC, W). All herbarium specimens consist of unattached material, either ball-shaped or free-floating entangled masses. The collections span a 110 year period (1852–1962). Lake balls of this kind have also been reported in the literature from the Beulaker Wijde (Koster 1959) and Duinigermeer (Segal and Groenhart 1967), both in the 'De Wieden' area, province Overijssel. All locations are listed with additional information in Table 1. None of the contacted monitoring organizations had ever recorded *A. linnaei*. While most reports of 'lake balls' by boat rental businesses could be instantly regarded as being cyanobacterial by how they were described, there was initially convincing information given by amateur divers about *Aegagropila*-like balls in recent years from the Lake 'De Groene Heuvels'.

Field work

The results of the field trips are summarized in Table 2. All historical locations of *A. linnaei* gave a similar impression with signs of eutrophication as

Table 3 List of specimens for which partial LSU rDNA sequences have been determined

Location	Lat.	Long.	Growth form	Collectors	Coll. date	No.	Voucher	GenBank accession no
Lake Myvatn, Iceland	N65°36′36″	E17°02′36″	Ball	Á. Einarsson	2002	B54	L0793580	EU655697
Ukraine (aquarium shop)			Ball		2005	C01	L0793577	EU655698
Holmön, Baltic Sea, Sweden	N63°46′48″	E20°53′24″	Attached filaments (on rock)	L. Bergström and J. Ask	2004	C41	L0793571	EU655702
Ramsholmen, Baltic Sea, Finland	N60°02′50″	E23°28′48″	Unattached, matted	R. Munsterhjelm	2007	L68	L0793579	EU655699
Sällvik, Baltic Sea, Finland	N60°01′48″	E23°30′00″	Unattached, matted	R. Munsterhjelm	2007	L69	L0793578	EU655701
Boven Wijde, The Netherlands	N52°43′48″	E06°06′36″	Attached filaments (on *Anodonta*)	C. Boedeker and A. Immers	2007	AI15	L0793572	EU655700

indicated by poor underwater light climate and organic muddy sediments, except the Naardermeer. Unattached mats or balls of *A. linnaei* were not encountered anywhere. Only in the Boven Wijde (province Overijssel), a single attached tuft of *A. linnaei* was found on a shell of the freshwater shellfish *Anodonta anatina* (Fig. 2). The filaments displayed all the typical morphological characters of the species (van den Hoek 1963; Leliaert and Boedeker 2007): subterminal insertion of branches (Fig. 2a–d), rhizoids in distal parts of the thallus (Fig. 2a), often opposite branches (Fig. 2b), irregular cell shape and unrestricted insertion of branches in basal parts (Fig. 2c), and serial insertion of branches (Fig. 2d).

The Naardermeer differed from the other sampling locations in having clearer water and a dense charophyte vegetation. Hundreds of unattached ball-shaped colonies of the cyanobacterium *Nostoc pruniforme* C. Agardh ex Bornet & Flahault were floating just above the bottom, resembling *A. linnaei* underwater (Fig. 3a, b).

The recreational Lake 'De Groene Heuvels', even though not a location from which the occurrence of *A. linnaei* had been reported earlier, was visited to verify reports of 'lake balls' by divers. Ball-shaped colonies of the ciliate *Ophridium versatile* (Müller) Ehrenberg and the cyanobacteria *Rivularia* sp., *Gloeotrichia pisum* (C. Agardh) Thuret, and *Tolypothrix polymorpha* Lemmermann were observed. The latter formed free-floating, dark-green tufts up to 4 cm in diameter (Fig. 3c, d), resembling *A. linnaei*. *A. linnaei* was not found and there is no evidence that the species ever occurred there.

DNA sequences

All six samples of *A. linnaei* (see Table 3), representing different growth forms, habitats, and locations showed identical nucleotide sequences in the LSU, except for one point mutation at position 178 (reference sequence: *Chlorella ellipsoidea* Gerneck, GenBank no.: D17810) in two samples. One of the two sequences of *A. linnaei* from the brackish Pojo Bay, Finland (L69, unattached populations) and the sequence from The Netherlands (AI15, attached) displayed an A instead of a G at that position. Close examination of the electropherograms at this variable position showed underlying peaks matching the

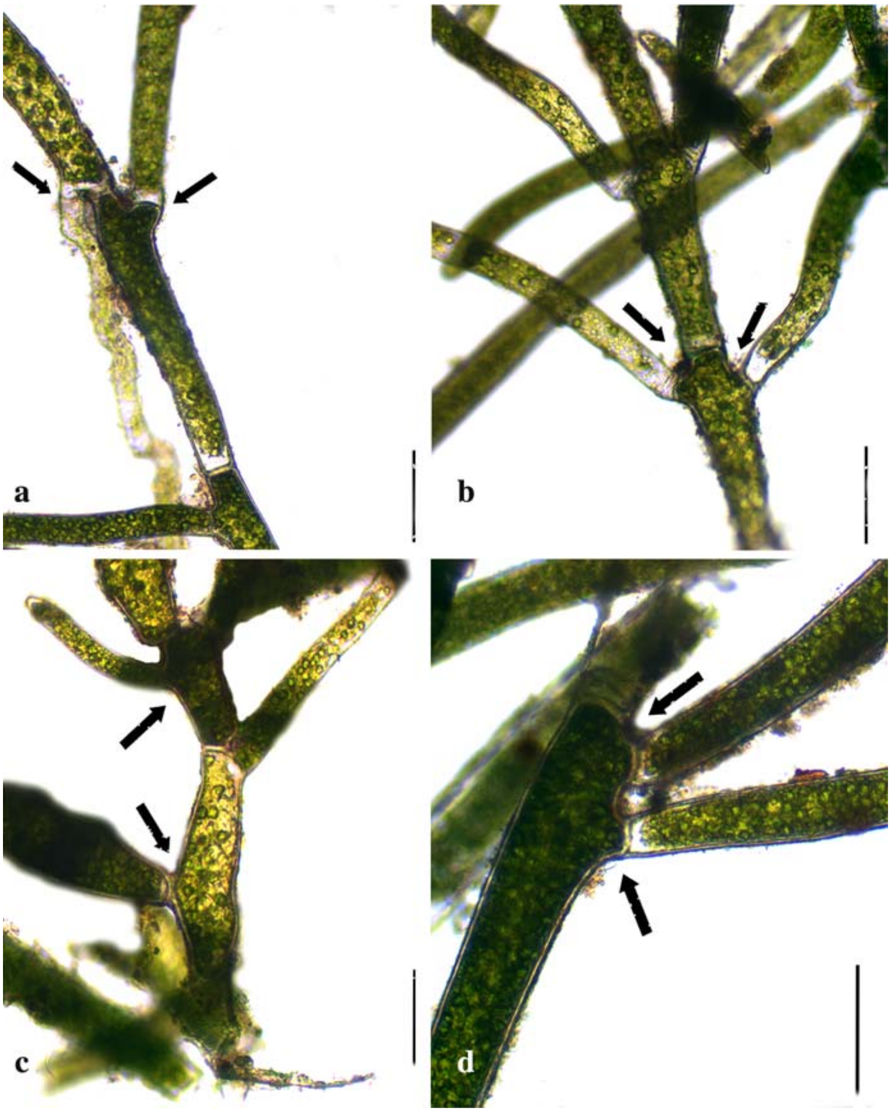

Fig. 2 Morphological characters of *Aegagropila linnaei* from the Boven Wijde, The Netherlands. **a** Subterminal insertion of branches and a rhizoid sprouting from the distal part of the thallus. **b** Subterminal insertion of branches, opposite branching, irregular cell shape. **c** Irregularly shaped cells in the basal region, branching from the middle of a cell, thick cell walls. **d** Serial insertion of branches. Scalebars = 100 μm

corresponding base of the other sequence in three out of the six sequences, suggesting that the observed differences from direct cycle sequencing might be attributable to intragenomic variation.

Discussion

There are no Dutch records of the enigmatic green alga *A. linnaei* dated later than 1962 preserved in any official herbarium. The last report of the species in the literature is dated 5 years later (Segal and Groenhart 1967). After 1967, there are no reports until our present study. During a survey of the collections of *A. linnaei* from 29 herbaria, only 9 out of more than 1,000 specimens had been collected in Europe in the last 30 years (data not shown). Even though this reflects a change in the tradition of collecting and depositing freshwater algal specimens during the last century to some degree, the obvious anthropogenic changes in the natural habitats most probably have led to a decline in populations in The Netherlands and elsewhere.

Field work

Searching for unattached algae floating over the bottom, such as *A. linnaei*, in peaty or eutrophicated

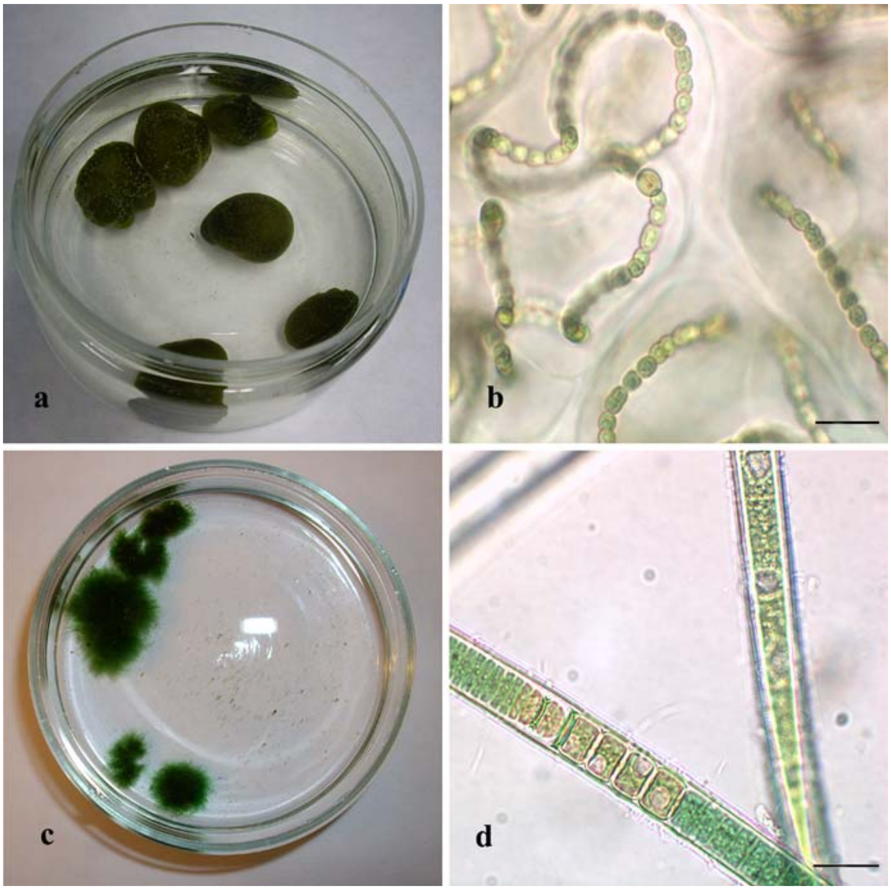

Fig. 3 Cyanobacterial 'lake balls', resembling *A. linnaei* underwater. **a**, **b** *Nostoc pruniforme* from the Naardermeer. **c**, **d** *Tolypothrix polymorpha* from 'De Groene Heuvels', Wijchen. Diameter of the Petri dishes = 10 cm, scalebars = 20 µm

lakes with poor visibility is very difficult. Although the lake balls have been frequently reported to occur in large numbers, the populations often seem to be restricted to a specific part of the lakes (Koster 1959; Pankow 1965; Kurogi 1980; Pankow and Bolbrinker 1984; Einarsson et al. 2004). In those areas, light supply, depth, sediment type, slope, and water movements obviously allow the unattached forms to thrive. Even when targeting typical habitats such as shallow sandy bottoms, reed belts, downwind bays or specifically mentioned historic collection sites, and deploying a range of methods, populations could be entirely missed. This is even more likely if the unattached forms (mats and balls) have disappeared and if the remaining population of attached individuals is small and patchy. Finding the alga on one single unionid mussel shell in one location may suggest a small and scattered population. *A. linnaei* has also been found attached on unionids in Japan (Niiyama 1989; Wakana et al. 2001, 2005). Growing on shellfish in shallow water might be one way how populations can survive when not enough light penetrates into deeper waters anymore due to eutrophication effects.

Growth forms and habitats

The range of different growth forms, the morphological plasticity of the species, and the range of habitats have led to the description of a large number of species and forms, but these were later synonymized by van den Hoek (1963). Lakes are generally regarded as the typical habitat of *A. linnaei*, and the species can be locally abundant or even dominant (van den Hoek 1963). Attached populations also occur in several rivers. The epilithic form has been found in the Seine and the Moine in France (van den Hoek 1963), in the river Sasso in Switzerland (van den Hoek 1963), the Fényes spring in Hungary (Palik 1963), in the Oslava river in the Czech Republic (as *Cladophora moravica* (Dvořák) Gardavský 1986), and in the rivers Tees, Tyne, Tweed, and Wear in the UK (Holmes and Whitton 1975; Whitton et al. 1998). Another very different habitat of *A. linnaei* is the brackish northern part of the Baltic Sea, where it grows mostly attached on rocks. *A. linnaei* is widespread and locally abundant in the Gulf of Bothnia (Waern 1952; van den Hoek 1963; Bergström and Bergström 1999), where the salinity is below 6 psu.

Two ball-shaped samples of *A. linnaei* from two lakes as distant as Japan and Sweden were previously shown to have identical small subunit (SSU) rDNA gene sequences (Hanyuda et al. 2002). Here, we present six partial sequences of the more variable large subunit (LSU) rRNA gene. These sequences represent the three different growth forms (ball-shaped, unattached mats, epilithic/epizoic), a range of habitats (brackish and freshwater) and distant locations (Table 3). All sequences are identical except for one shared point mutation in the sample from The Netherlands and in one of the samples from Finland, confirming that *A. linnaei* has a wide distribution and spans a range of habitats. Therefore, it seems justified to refer to all growth forms as one species. An isozyme study of *A. linnaei* populations in Japan revealed genetic differentiation and limited gene flow between attached and unattached populations, emphasizing the need for conservation efforts for all growth forms (Soejima et al. 2008).

Eutrophication in The Netherlands

All lakes in The Netherlands where *A. linnaei* had been collected in the past were severely eutrophicated, starting around the 1960s and 1970s. Characteristic for these eutrophicated systems were the reduction in water transparency and the absence of charophyte vegetation (Leentvaar and Mörzer Bruijns 1962; Maasdam and Claassen 1998; Riegman 2004; Boosten 2006; Kiwa Water Research 2007; Waternet, personal communication; W. Fryslân, personal communication). Restoration measures undertaken in the 1990s led to improved water quality and a return of charophyte vegetation only in the Duinigermeer (van Berkum et al. 1995), the Zuideindigerwiede (E. Nat, personal communication), and the Naardermeer (Boosten 2006), while restoration attempts have not improved the situation in the Loosdrechtse Plassen so far (Hofstra and van Liere 1992; Van Liere and Gulati 1992).

From the herbarium survey of locations worldwide and the potential natural state of those lakes, as well as from many literature reports, it is apparent that *A. linnaei* typically occurs in oligo to mesotrophic lakes, but can also occur in dystrophic and slightly eutrophic habitats. Only the attached form has been encountered sporadically in eutrophic or disturbed habitats, such as the rivers Seine and the Moine (van den Hoek 1963) or the Boven Wijde. Due to self-shading, the unattached forms need good light conditions to grow (Yoshida et al. 1994), and they might therefore be more sensitive to eutrophication due to the invariable deterioration in underwater light climate. A reduction in light availability has been shown to cause the decline in charophytes in eutrophicated lakes in Sweden (Blindow 1992). Another negative effect on submerged vegetation in eutrophicated waters can be damage by bottom feeding fish such as bream (ten Winkel and Meulemans 1984). In the case of *A. linnaei*, the change from sandy to muddy sediments in eutrophicated waters would have a negative impact, especially on the formation of balls. In addition to eutrophication, changes in hydrology and turbidity could also have a strong negative effect on unattached growth forms.

It is most likely that the populations of *A. linnaei* died out during the 1960s and 1970s in the Naardermeer, the Loosdrechtse Plassen, the Zuideindigerwiede, the Duinigermeer, the Molengat, and the Zwarte Broek, because of the effects of eutrophication. The finding of the species in the eutrophicated Boven Wijde might represent the remainder of a once larger population. An alternative, less likely, explanation is that *A. linnaei* could have become extinct everywhere in the 1970s and could have recently recolonized the Boven Wijde. There is no indication that habitats with an improved water quality such as the Naardermeer have been recolonised, and the recolonisation potential of the species might be very low. The restored Naardermeer seems like a suitable habitat for *A. linnaei*, as judged by the trophic level (mesotrophic), the charophyte vegetation, and especially the presence of *Nostoc pruniforme* (see Mollenhauer et al. 1999). Dispersal by water birds should theoretically make recolonization of suitable habitats possible (e.g., Schlichting 1960). On the other hand, the assumed small size of source populations, the rarity or complete absence of sexual reproduction (Soejima et al. 2008), and the very slow growth rates (van den Hoek 1963; personal observation.) reduce the likelihood of re-establishing populations. Fragmentation can be regarded as an efficient way of dispersal in some groups of algae. However, fragmentation as the only means of reproduction in a slow-growing organism such as *A. linnaei*, i.e., without any form of additional spore release, results in a low dispersal potential.

Decline and threat of *Aegagropila linnaei*

Aegagropila linnaei is seemingly extinct in most of the original locations in The Netherlands, where this species used to be quite abundant (see Table 2), as already reported for the Zuideindigerwiede (Segal and Groenhart 1967). Extinct populations have also been reported in the literature from the German Baltic Sea coast (Schories et al. 1996) and from Lake Galenbecker in northeastern Germany (Pankow 1985). In some instances, the ball form became extinct while the epilithic filamentous form still persisted, i.e., in Lake Zeller in Austria (Nakazawa 1974; Kann and Sauer 1982) and in Lake Akan in Japan (Wakana 1993; Wakana et al. 1996). Also this seems to be true for our study. A decline in population numbers and/or size is known from Lake Myvatn in Iceland (Einarsson et al. 2004), from Takkobu Marsh and other small swamp lakes in Japan (Wakana et al. 2001, 2005), and from Denmark, where the only population left occurs in Sorø Sø, Sjæland (R. Nielsen, personal communication). The observed decline in populations or the natural habitats being under threat led to the inclusion of the species in several national red lists or other conservation instruments. *A. linnaei* has a status as an endangered or protected species in Japan (Environment Agency of Japan 2000), Iceland (Á. Einarsson, personal communication), United Kingdom (provisional, Brodie et al. 2008), Germany (Ludwig and Schnittler 1996; Schories et al. 1996), Sweden (Gärdenfors 2005), Estonia (Lilleleht 1998), and Russia (Noskov 2000).

Eutrophication of aquatic systems is a common process worldwide and leads to the loss of unique habitats and a reduction in biodiversity (i.e., Bayly and Williams 1973). Restoration of affected water bodies once polluted is difficult, costly, and takes a long time. Even after restoration, ecosystems do not necessarily return to their original diversity and community structure (Entwisle 1997). If a habitat is successfully recolonized by *A. linnaei*, it might take decades before populations abundant enough will have built up to develop into the typical unattached mats or the lake balls. On the other hand, there is some indication for genetic differentiation between unattached and attached populations, as well as between different attached populations (Soejima et al. 2008). Therefore, loss of genetic diversity within *A. linnaei* might have already occurred in The Netherlands. The ball shapes that *A. linnaei* can produce have led to its popularity among scientists and naturalists, as well as in the aquarium trade, and Japanese society. This popularity could be the key to its conservation, and *A. linnaei* could possibly function as a flagship species of endangered freshwater algae other than desmids or charophytes. The situation in The Netherlands would certainly justify the inclusion of this species in a red list of The Netherlands or similar conservation instruments. At the moment, red lists only exist in The Netherlands for animals, fungi, vascular plants, and lichens. Red lists are possibly a first step in conservation measures, but it is impossible to protect individual algal species, thus only conservation of the natural habitats and catchment management could be successful. The future of *A. linnaei* in The Netherlands, and in some other regions, is uncertain.

Conclusions

Aegagropila linnaei had not been found in The Netherlands for more than 40 years, but is not extinct since the species was encountered again during a recent field survey, for the first time in the attached form. In all but one of the original locations, the species could not be found anymore. Taking into account the history of eutrophication in these lakes, it must be assumed that the species became extinct in those locations during the 1960s and 1970s. Unattached growth forms such as the enigmatic lake balls do not occur in The Netherlands anymore. Some of the original habitats have been restored and are candidates for possible recolonization, but the species might have a poor recolonizing potential. We propose to include this species in a national red list. In addition, it was shown that specimens with different growth forms from different locations and habitats have basically identical partial LSU rDNA sequences and either represent one single species or a complex of closely related cryptic species.

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