CO₂ sequestration by propagation of the fast-growing *Azolla* spp.

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Abstract

Azolla is a group of aquatic floating plants that can achieve very high growth rates compared to other aquatic macrophytes, with a doubling time of 2–5 days under optimal growing conditions. The ability of *Azolla* to grow at such rapid rates allows for the opportunity of utilizing it as a method to sequester a significant amount of atmospheric CO₂ in the form of biomass, which can be locked away to completely remove the carbon from the active carbon cycle, or which can be used in various applications such as animal feeds, biofertilizers, and biofuel production, which in turn will contribute to reduction in the fossil CO₂ emissions. In this desktop study, the potential use of *Azolla* for mitigating the annual increase in the atmospheric CO₂ levels was addressed, which were estimated at 18.9 billion tons of CO₂ per year. A theoretical setup of 1-ha ponds was assessed to estimate the total *Azolla* growing area required for counterbalancing the annual atmospheric CO₂ increase. Each 1-ha pond was found capable of capturing 21,266 kg of CO₂ (C) per year. The calculated required total area to mitigate the total annual increase was estimated to be 1,018,023 km² (equivalent to around a fifth of the Amazon forest area). Sensitivity analysis, which was based on the variations in the productivity of *Azolla* due to growing conditions, indicated that the required area would range between 763,518 and 1,527,036 km². This study provides a novel natural method for CO₂ sequestration that has lower environmental impacts compared to conventional sequestration technologies as an alternative green approach for mitigating the effects of fossil fuels.

Keywords *Azolla* · Carbon dioxide (CO₂) · Global warming · Greenhouse gas · CO₂ sequestration

Introduction

What is *Azolla*

Azolla is a genus comprising a group of aquatic floating plants, namely ferns, which are mainly native to warmer climates such as the tropics and subtropics, as well as to the warm temperate regions of Africa, Asia, and the Americas (Bocchi and Malgioglio 2010; Sadeghi et al. 2012; Wagner 1997). Given its sensitivity to the lack of water, growth of *Azolla* is limited to aquatic ecosystems such as stagnant waters and ponds, which are usually covered by mats of *Azolla* along with other associated species of floating plants like duckweeds, water lettuce, water caltrop, and water meal

(Sadeghi and Zarkami 2013; Wagner 1997). What drives the attention towards *Azolla* among other similar species is being one of the fastest growing aquatic macrophytes, reaching a remarkable doubling time of only 2–5 days based upon the growth conditions, compared to many widely distributed aquatic macrophytes such as the relatively rapidly growing bryophyte *Ricciocarpos natans* (L.) Corda, which can achieve a doubling time of around 27 days (Brouwer et al. 2018; Gimenes et al. 2020; Hove 1989; Katole et al. 2017; Sadeghi and Zarkami 2013; USDA 2021). Given some of the growing characteristics of *Azolla*, such as its fast growth and tolerance to pollution, *Azolla*, in many cases, is considered an annoying weed that can sometimes turn into an invasive plant in certain ecosystems, especially in wetlands, although it offers extensive benefits, such as being used as a biofertilizer for crops or a feed for cattle and even as a feed in aquaculture (Bocchi and Malgioglio, 2010; Katole et al. 2017; Mosha 2018; Mvandaba et al. 2019; Pinero-Rodríguez et al. 2019).

The massive growing capability of *Azolla* is mainly due to its ability to grow even in the absence of any nitrogen in

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the water. *Azolla* has free access to atmospheric nitrogen fixation through its complex symbiotic relationship with *Anabaena azollae*, which is an endophytic blue-green alga that lives within cavities in the leaves of the *Azolla* macrophytes (Bocchi and Malgioglio 2010; Gresshoff 2018; Hove 1989). *Anabaena* can provide sufficient nitrogen for itself as well as for the *Azolla* plants, allowing for optimal growth of *Azolla* under nitrogen limiting conditions, thus contributing significantly to the rapid proliferation of the water fern. One famous example of the benefits of nitrogen fixation by *Azolla* is supplying nitrogen to the rice ecosystems. *Azolla*, which requires shady conditions for optimal growth, proliferates optimally in the shade of the rice plants while simultaneously providing nitrogen for proper growth of the rice (Yao et al. 2018). Sometimes *Azolla* can be used as a biofertilizer for a variety of crops due to its high biomass productivity (Hanafy et al. 2018; Kaur and Purewal 2019). It can be also used to control high nitrogen and phosphorus levels in contaminated freshwater sources in certain polluted ecosystems (Amare et al. 2018; Wang et al. 2019). The capability of *Azolla* of rapid growth is heavily aided by its tolerance to a variety of stressful environmental conditions. These stresses include changes to pH, nutrient levels in the water, and accumulation of pollutants within the water (Arora et al. 2006; Hanafy et al. 2018; Sadeghi and Zarkami 2013; Sarkar and Jana 1986).

Types of *Azolla*

There are 6 living species of *Azolla* comprising two subgenera, with the subgenus *Euazolla*, containing *Azolla filiculoides* Lam., *Azolla caroliniana* Willd., *Azolla mexicana* Schlttdl. & Cham. ex C. Presl, and *Azolla microphylla* Kaulf., and the subgenus *Rhizosperma* containing *Azolla pinnata* R. Br. and *Azolla nilotica* Decne. ex Mett. (JSTOR 2021; Sadeghi and Zarkami 2013; USDA 2021).

A. nilotica is native to East Africa and can be easily identified compared to other species by its larger size when grown in favorable conditions, reaching several tens of centimeters. *A. pinnata* can be found in areas of South Africa, Australia, Madagascar, and subtropical and tropical Asia. *A. caroliniana*, *A. mexicana*, and *A. microphylla* can be found in many areas of the temperate, subtropical and tropical regions of the Americas. *A. filiculoides* is native to America and East Asia such as in New Zealand and Japan (Mosha, 2018; Sadeghi and Zarkami 2013). Figure 1 shows the wide distribution of *Azolla* across the world.

Characteristics of *Azolla* macrophytes

Macrophytes of *Azolla*, also known as fronds, usually range in size from 1 to 2.5 cm, and can reach lengths of about 15 cm in some species. The algal symbiont resides in the cavities of the leaves of the fern (Wagner 1997).

Azolla macrophytes are characterized by their high biomass productivity due to the much higher photosynthesis rates compared to most C4 plants. *Azolla* macrophytes can double in mass within less than 5 days even in a nitrogen free medium (Wagner 1997). Under optimal conditions, *A. filiculoides*, *A. caroliniana*, *A. mexicana*, and *A. pinnata* possessed a doubling time of 2 or less days (Brouwer et al., 2018; Hanafy et al. 2018; Sadeghi and Zarkami 2013; Wagner 1997). Various studies reported that the maximum biomass attained by *Azolla* ranges from 64 for *A. pinnata* to 520 g dry weight/ m² in *A. filiculoides* (Wagner 1997). Growth rate of *Azolla* was also reported to range around 25 to 90 g/m²/day in *A. nilotica*, reaching a maximum biomass of 2900 g/m² of water, which is considered very high in this species in particular due to the larger size of the fronds and their tendency to extend vertically (Wagner 1997).

Fig. 1 Occurrence (incidence) and distribution of *Azolla* species (adapted from GBIF 2020)

CO₂ sequestration: importance of Azolla

The latest reports indicate that the annual CO₂ emissions into the atmosphere are estimated to be around 36 billion tons of CO₂ per year (“CO₂ and Greenhouse Gas Emissions,” 2019). There are several natural sinks for carbon dioxide that are extremely important to balance this huge amount of carbon that is released into the atmosphere, mainly through photosynthetic sequestration in plants, and through absorption into the oceans. Such processes were reported to contribute to the removal of around 45–60% of the annual CO₂ emissions during the last few decades (Ballantyne et al. 2012). This indicated that during the first decade of this century, the annual atmospheric growth rate of atmospheric CO₂ was almost constant at around 4 billion tons of carbon dioxide per year, with Keenan et al. (2016) further reporting that there is no increase to the annual CO₂ growth rate since then, therefore contributing to a linear increase in the atmospheric CO₂ levels of around 2 ppm per year since 2002. This increase is a balance between the global generation rates of CO₂ in relation to its sequestration rate. Thus, there is a need to mitigate this deficit to at least maintain the current level of atmospheric CO₂, or even trying to go back to the significantly lower levels which were prevalent previously at around 320 ppm during the 1960s.

Since the industrial revolution, the amount of carbon in the atmosphere (mainly in the form of CO₂) has increased significantly due to anthropogenic activities, which released carbon from other reservoirs into the atmosphere at a rate that is higher than the rate of removal of atmospheric carbon. This is mainly because of the use of fossil fuels accompanied by significant deforestation. It was estimated that around 410 billion tons of carbon have been released into the atmosphere from fossil fuels between 1750 and 2015, which contributed to a significant increase in the atmospheric burden of CO₂ by around 260 billion tons of carbon over that period of time (Ussiri and Lal, 2017). This has led to major environmental impacts due to global warming and climate change, which contributed to other environmental and human health issues since this rise started. Major examples of such impacts include extreme weather events like extended droughts and floods, wildfires, an increase in the number of disease-carrying vectors, and the rise of associated vector-borne diseases (Miller and Spoolman 2019).

CO₂ cycling involves both biotic and abiotic processes that transfer the carbon between the natural carbon reservoirs through complex natural and anthropogenic interactions, with the carbon cycle being in a steady state if anthropogenic contribution was excluded (Ussiri and Lal, 2017). Such processes include aquatic systems and terrestrial systems. In aquatic systems, oceans are the major sink for CO₂, especially on the long term, as the oceans can absorb much of the released atmospheric CO₂ through equilibration

with the carbonate buffer system. This will ultimately shift the equilibrium towards alkalinity, which can be ultimately incorporated as calcium carbonate into various aquatic inhabitants such as plankton. On the long term, this absorbed carbon will sink to the bottom of the oceans in the form of dead biomass, where it can be locked away from being rereleased into the atmosphere. Assimilation by terrestrial ecosystems is also an important factor in the natural carbon cycle, which is mainly due to fixation by plants. The majority of this assimilation occurs in large terrestrial ecosystems around the world, which includes forest ecosystems, in which plants can act as a sink for atmospheric CO₂, mitigating a major portion of the anthropogenic carbon emissions (Favero et al., 2020). A very important consideration in the case of this plant assimilated CO₂ is that the carbon might be released back to the atmosphere due to human activities, such as through cutting and burning of these forests. Hence, utilizing plants can be an effective way of assimilation of atmospheric carbon, mainly if the sequestered carbon was stored away and was not allowed to be released back into the atmosphere. Furthermore, an important consideration to using plants to sequester the atmospheric carbon is that although the biomass might be utilized in ways that might release this carbon back into the atmosphere, such as using the biomass as a feed or as a biofuel; however, the assimilation will indirectly contribute to lowering the input of additional carbon being pumped into the atmosphere from fossil fuels, which are the major reason for the release of locked carbon into the atmosphere.

It was estimated that around 20–30% of the anthropogenic CO₂ emissions are absorbed by terrestrial ecosystems annually (Ussiri and Lal, 2017). Elevated CO₂ concentrations also contribute to CO₂ fertilization effect, in which there is an increase in the photosynthesis rates of plants, thus contributing to a faster assimilation rate of CO₂ (Borys et al., 2016; Yang et al. 2016). However, the accelerating CO₂ emissions, combined with disruption of major natural CO₂ sinks through destruction of forests around the world, is the major reason behind the continuous increase in the atmospheric CO₂ levels. This is mainly due to imbalances in the carbon cycle resulting from failure of environmental sinks to keep up with the emissions, therefore leading to harder control over stabilization of the atmospheric CO₂ concentrations.

The uncontrolled rise in the atmospheric CO₂ levels can be mitigated in two major ways, by controlling and minimizing the emissions resulting from fossil fuels, and by removing/transferring atmospheric CO₂ through different means into other securely storable carbon pools, a process known as CO₂ sequestration (Ussiri and Lal, 2017). There are various CO₂ sequestration methods, which include abiotic as well as biotic processes. Abiotic processes involve the capture of CO₂ and its subsequent storage in locations which prevent

its escape into the atmosphere, such as deep underground (Benson and Surles 2006). Biotic processes on the other hand take advantage of the natural ability of plants to absorb CO₂ by transforming it into biomass, which in turn can be utilized in a variety of useful applications such as animal feeds and production of biofuels (Brune et al., 2009; Rollins et al. 2002). Therefore, biotic carbon sequestration can be an efficient method to balance the global carbon budget, at least to combat the annual increase in the CO₂ concentration through assimilation of the CO₂ deficit.

The capability of *Azolla* to grow very rapidly under optimal conditions provides an opportunity to sequester some of the CO₂ being released into the atmosphere each year, therefore providing an opportunity to reduce global warming. Generally, *Azolla* grows optimally under shade (15–18 Klux), moderate temperatures (18–28 °C), and moderate to high relative humidity (55–83%) (Sadeghi and Zarkami, 2013). Optimum growing requirements of *Azolla* spp. are detailed in the [supplementary information](#). Actually, during Earth's history, mass abundances of *Azolla* remains were accumulated sporadically in the Eocene Arctic and Nordic Seas, which dated to around 48.5 million years ago and were referred to as the *Azolla* phase, and coincided with a significant drop in the global CO₂ levels. They marked the onset of the transition phase from the greenhouse Earth to the icehouse Earth (Speelman et al., 2009). During this time, *Azolla* acted as a sequesterer for atmospheric carbon, mainly by sedimenting and locking of the carbon away after fixing it from the atmosphere, storing an estimate of 0.9×10^{18} to 3.5×10^{18} g of carbon, which translates into a 55- to 470-ppm drop in the atmospheric CO₂ levels. Continuous growth of the fern, combined with sedimentation of dying biomass that settled into the sediments of the oceans, contributed to $0.9\text{--}3.5 \times 10^{15}$ kg of stored carbon, aiding substantially in decreasing the levels of atmospheric CO₂ (Speelman et al., 2009).

With the huge contribution of humans to global warming, mainly due to the increased CO₂ emissions, it is important to try to mitigate the global carbon footprint. As per the latest estimations, the global fossil CO₂ emissions averaged around 9.4 billion tons of carbon per year during the last decade, with some estimations showing that the annual total emissions ranged around 36 billion tons of CO₂ (equivalent to 9.8 billion tons C) per year shortly before the COVID-19 pandemic that affected the global emissions (Friedlingstein et al., 2020; Ritchie and Roser 2017). This mitigation can be achieved either by reducing the production of CO₂, as in the case of alternative energy sources to fossil fuels, or by sequestering significant amounts of CO₂, as in the case of planting sufficient amounts of trees. Historically, *Azolla* has contributed significantly to the regulation of the climate of Earth through

assimilation of significant amounts of atmospheric carbon, and hence, *Azolla* still has the potential to be utilized once again to mitigate the current rise in the atmospheric CO₂ levels. There is a lot of research targeting the use of *Azolla* and understanding its growth for various purposes: utilizing the biomass as a biofertilizer, animal feed, treatment of wastewater, and phytoremediation of various pollutants such as heavy metals (Arora et al. 2006; Brouwer et al. 2018; Hove 1989; Katole et al. 2017; Mosha 2018; Yao et al. 2018). Its assessment for capturing atmospheric CO₂ is yet to be addressed. This paper thus addresses the theoretical potential use of *Azolla* for offsetting the annual increase in the atmospheric CO₂ levels in terms of the required *Azolla* biomass to be grown as well as the required area needed to support its growth.

Materials and methods

Growth equations

Understanding the growth of *Azolla* under optimal conditions requires knowing the growth model of the increase in biomass. Brouwer et al. (2018) showed that the growth of *Azolla* follows an initial exponential phase, followed by a transition to a linear growth pattern when the fern reaches standing crop conditions. The standing crop conditions occur when the density of the *Azolla* reached around 30 to 60 g/m² (dry weight). During the exponential growth phase, the relative growth rate (RGR) of *A. filiculoides* and *A. pinnata* was reported to be 0.337 d⁻¹ for the former and 0.317 d⁻¹ for the latter (Brouwer et al., 2018). It was also indicated that under optimal conditions, the RGR can easily reach 0.5 d⁻¹, corresponding to a biomass doubling time of 2 days (Brouwer et al., 2018).

The following equations describe the growth of *Azolla*. Equation 1 represents the exponential growth equation and Eq. 2 represents the linear growth equation as described by Brouwer et al. (2018).

$$y_t = y_0 e^{RGR \cdot t} \quad (1)$$

$$y_t = AGR \cdot t + b \quad (2)$$

where y_t is the standing crop (g/m²); y_0 is the initial standing crop (g/m²); AGR is the absolute growth rate (g/m²/day); RGR is the relative growth rate (per day); b is the standing crop at the start of the linear growth (g/m²); and t is the time (days).

AGR was reported by Brouwer et al. (2018) to be 11.9 ± 14 and 11.1 ± 7 g/m²/day for *A. filiculoides* and *A. pinnata*, respectively.

Summary of the optimal growing conditions for *Azolla*

Table 1 represents a summary of the optimal growth conditions for *Azolla*, which must be considered when choosing an appropriate location to sustain maximal growth rate of the fern and for a large-scale *Azolla* cultivation project. Few studies mentioned the requirements for specific species of *Azolla*, while most studies focused on optimal growth conditions for *Azolla* in general without indicating the species. No information was found for *A. nilotica* and *A. microphylla* with respect to the reported parameters in the table.

Therefore, if maximum harvest size of *Azolla* was targeted, the main criterion for collection of the biomass is the maximum productivity point (highlighted in Fig. 2), which is attained at some point after the transition to the linear growth phase, assuming that the production of *Azolla* was performed in cycles of growth starting with a seed biomass amount. If continuous harvest of *Azolla* was targeted, it would be better to keep the *Azolla* growing in the exponential phase, therefore periodically doubling the biomass until it reaches the standing crop. At this point, enough biomass will be harvested to free up space for the *Azolla* to maintain its periodic doubling rather than entering the linear growth phase.

CO₂ uptake

Available data about the rate of CO₂ consumption by *Azolla* are limited. Cheng et al. (2010), who studied the response of *A. filiculoides* to elevated CO₂ levels, reported that the CO₂ assimilation rates of *Azolla* varied between 47.1 and 142 mg C/pot under ambient CO₂ levels in a pot area of 300 cm², which lasted for 28 days under exponential growth conditions. This corresponds to 56–169 mg C/m²/day. Yet, there is significant research reporting the growth rate of *Azolla* in terms of biomass accumulation rates under optimal conditions, which can be correlated to the CO₂ assimilation in the biomass.

Several studies reported that the carbon content of *Azolla*, irrespective of the species and irrespective of the growing conditions, is fairly constant, ranging from 39 to 44% of dry weight of the harvested plants (Bocchi and Malgioglio, 2010; Cheng et al. 2010; Speelman et al. 2009). Knowing the carbon content of *Azolla*, the carbon assimilation rate of the *Azolla* would thus be easily calculated. The calculations assume an average carbon content of 42%.

Azolla/CO₂ sequestration setup

Assuming that upon choosing a suitable location with enough surface area for *Azolla* to grow without reaching the standing crop conditions, the fern will be thus sustained

under exponential growth conditions, enabling it of reaching its maximum growing capacity of less than 2 days for each doubling. Such a setup will allow for high rates of CO₂ sequestration by continuously growing and harvesting *Azolla* at regular intervals to maintain the minimum possible doubling time.

The proposed setup for CO₂ sequestration in this study will be based per 1 ha, which will be used for calculating the annual performance. This setup will enable the calculation of the number of ponds required for complete assimilation of the annual CO₂ addition of 18.9 billion tons of CO₂ per year or 5.15 billion tons of carbon (1 CO₂ = 0.27 C).

The proposed setup will assume that at the start of operation, the pond will have already reached its maximum exponential growth rate, excluding the startup culturing of *Azolla* in the pond. The setup will consider that enough *Azolla* is present in the pond to reach its full capacity at the end of the doubling time of the *Azolla* species before reaching the critical standing crop density of an average of 450 kg/ha, when the growth will shift to a linear increase. Wagner (1997) reported that *A. filiculoides*, *A. caroliniana*, *A. mexicana*, *A. pinnata*, and *A. nilotica* can all reach a doubling time of 2 days under the optimal conditions. The standing crop density, considering the standardized area of 1 ha, will also represent the biomass production of *Azolla* that is enough to cover that area every 2 days (the doubling time). Assuming this doubling time of 2 days, *Azolla* will thus be harvested every other day when it reaches 450 kg/(ha). Enough *Azolla* will then be harvested to leave the minimum amount that is required to reach 450 kg at the end of each of the growth/harvest cycle.

Results

Residual annual atmospheric carbon dioxide

Assuming an average annual CO₂ removal of 52.5% (Keenan et al. 2016; Knorr 2009), and taking the annual CO₂ emissions around 36 gigatons of CO₂ per year into consideration, the current estimation of annual CO₂ increase would be around 18.9 gigatons of CO₂ per year. Therefore, *Azolla* will have to be cultivated to sequester around this amount.

Productivity of *Azolla* and growth characteristics

The combination of the various growing conditions affects the growing rate and the productivity of *Azolla*. Given availability of stable conditions (light intensity, temperature, pH, etc.), growth of *Azolla* follows a sigmoid (S-shaped) pattern in several distinct phases as described by Hove (1989). As *Azolla* is added to a growing pond, it utilizes the available resources to grow exponentially, namely due to the absence

Table 1 Literature compilation of *Azolla* spp. optimal growth conditions

Parameter	Available information	Synthesized range
Water depth (cm)	<ul style="list-style-type: none"> ➤ < 5 cm (Sadeghi and Zarkami, 2013) ➤ 5–10 cm (Hove 1989) ➤ 30–120 cm (Sadeghi et al., 2012) ➤ 50 cm (Sadeghi et al. 2012) 	➤ 5–120 cm
Thickness of <i>Azolla</i> layer	➤ 2–3 cm (Sadeghi and Zarkami, 2013; Wagner 1997)	➤ 2–3 cm
Photoperiod	<ul style="list-style-type: none"> ➤ 20 h (Wagner 1997) ➤ 5 h (Sadeghi et al., 2012) 	➤ 5–20 h
Light intensity	<ul style="list-style-type: none"> ➤ 15–18 klx (25–50% full sun) (Sadeghi and Zarkami, 2013) ➤ 50 klx (50% full sun) (Wagner 1997) ➤ 50 klx (50% full sun) (Hove 1989) ➤ Nitrogen fixation decreases significantly at light intensities lower than 10–13 klx (Bar et al. 1991; Costa et al. 2009) 	➤ 10–18 klx
pH	<ul style="list-style-type: none"> ➤ 4.5–7 (Wagner 1997) ➤ 5.3–5.8 (Katole et al., 2017) ➤ 4 to 10 (Hove 1989) ➤ 7.63 ± 0.31 (Sadeghi et al., 2012) ➤ Optimal pH depends on temperature, nutrients, and light intensity (Wagner 1997) 	➤ 4–10
Humidity (%)	<ul style="list-style-type: none"> ➤ 70–75 (Sadeghi and Zarkami, 2013) ➤ 85–90 (Wagner 1997) ➤ 75 ± 4.5 (Sadeghi et al., 2012) ➤ 7.8 ± 4.5 (Sadeghi et al. 2012) ➤ High humidity (> 80%) hinders optimal biomass production (Sadeghi and Zarkami, 2013) 	➤ 7.8–90%
Temperature °C	<ul style="list-style-type: none"> ➤ 18–22 (Sadeghi and Zarkami, 2013) ➤ 18–28 (Wagner 1997) ➤ 25–35 (Katole et al., 2017) ➤ 25 (Hove 1989) ➤ 16.2 ± 6.8 (Sadeghi et al., 2012) ➤ Severe inhibitory effect below –4 °C and above 30 °C, with certain species being tolerant to frost (Ex. <i>A. mexicana</i>) (Hove 1989; Katole et al. 2017; Sadeghi et al. 2012) ➤ Maximum plant density (<i>A. caroliniana</i>) between 15 and 20 °C and highest biomass productivity between 20 and 30 °C (Debusk and Reddy, 1987) 	➤ 15–35 °C
Nitrogen	<ul style="list-style-type: none"> ➤ No nitrogen limitations due to the ability of <i>Azolla</i> to fixate atmospheric nitrogen ➤ Nitrogen fixation up to 4.6 kg N/ha/day (Kulasooriya et al., 1982) ➤ Nitrogen fixation of 0.4 to 3.6 kg N/ha/day, which varies based on presence/absence and concentration of nitrogenous nutrients (Kushari and Watanabe, 1992; Roger and Ladha 1992) 	➤ 0.4 to 4.6 kg N/ha/day
Phosphorus	➤ 0.3 and 1 mg/L for optimal growth (Kushari and Watanabe, 1992)	➤ 0.3 and 1 mg/L
Macronutrients and micronutrients	<ul style="list-style-type: none"> ➤ Rapid growth requires sustained presence of macronutrients such as potassium, calcium, and magnesium, which can be externally provided (Serag et al., 2000) ➤ Sustained growth requires micronutrients such as iron, molybdenum, manganese, zinc, copper, and cobalt (Biswas et al. 2005; Singh et al. 2010) ➤ High concentration of micronutrient could be inhibitory (Jain et al. 1992) ➤ Fe, Mn, Mo, and B: 50, 20, 0.3, and 30 µg/L, respectively (Wagner 1997) 	➤ Trace amounts in the range of µg/L
Salinity	<ul style="list-style-type: none"> ➤ High sensitivity to elevated salinity in the water (200 mM) (Thagela et al., 2018, 2017) ➤ 10–40 mM NaCl inhibits growth different <i>Azolla</i> species (Masood et al. 2006) 	➤ 10–40 mM NaCl
Pesticides and biological limiting factors	<ul style="list-style-type: none"> ➤ Highly sensitivity to chemical herbicides (Prasad et al. 2016; Silva et al. 2016) ➤ <i>Azolla</i> can become infected by bacteria, fungi, and viruses, limiting its growth and hindering its nitrogen fixation capability (Barreto et al. 2000) ➤ Insects pose a huge hazard to growth of <i>Azolla</i>, such as the weevil <i>Stenopelmus rufinasus</i> (Shaw et al. 2018) 	➤ NA

of any plant density restrictions. This enables the *Azolla* to take advantage of the available free space on the surface of the water, allowing for free dispersal of the new fronds without any constraints (phase I). This phase persists as long

as enough surface area is available for *Azolla* to spread out, until it covers all of the surface, thus reaching the standing crop density. After reaching the standing crop density, the growth transitions to a linear increase (phase II). This

transition is characteristic of each *Azolla* species, which was reported to reach up to 10,000 kg/ha (Hove 1989), but is generally much lower as found experimentally by Brouwer et al. (2018), being around 300–600 kg/ha. The linear growth phase continues until the *Azolla* approaches the maximum plant density, when the rate of increase in the biomass starts slowing down as the fronds of *Azolla* become more and more crowded (phase III). This continues until reaching the maximum possible plant density, after which the increase in biomass stops, being a factor of the growth of new tissue growth and loss of older fronds (phase IV). After that, phase V follows during which the population will slowly degenerate and die back. Assuming that the growth cycle of the *Azolla* was started from phase I (a new growth cycle) and left to reach the final stages, maximal productivity (kg/ha/day) is usually attained during the linear growth phase (phase II) at the point when the growth starts transitioning into slower rates approaching phase III. As the *Azolla* growth reaches this equilibrium, the productivity goes down significantly until it becomes minor without any noticeable increase in harvestable biomass.

The phases of *Azolla* growth are presented in Fig. 2.

Table 2 represents the various maximum crop densities that can be attained by various *Azolla* species (Wagner 1997), which are important to consider when monitoring the *Azolla* growth to maximize its growth without reaching physical limits that will compromise the increase in the *Azolla* biomass. It also presents the growth rates of various *Azolla* species that were reported to be grown under optimal conditions.

Azolla/CO₂ sequestration setup

To find the minimum required amount, the exponential growth equation will be applied to find y_0 , with $y_t = 450$ kg/

ha, $t = 2$ days, and $RGR = 0.5/\text{day}$ (corresponding to the doubling time of 2 days).

Given the exponential growth equation $y_0 = y_t / e^{RGR \cdot t}$, y_0 can be calculated. The standing crop y_0 will be thus 166 kg/ha. Therefore, every other day, the amount of *Azolla* that can be harvested will be the difference between the biomass at the end of the growth cycle and the amount to be left in the pond after harvesting: $450 \text{ kg/ha} - 166 \text{ kg/ha} = 284 \text{ kg/ha}$.

As a result, we will be able to sequester carbon dioxide that is equivalent to 284 kg of *Azolla* every 2 days/ha. This is equivalent to 50,633 kg of *Azolla* (dry weight) per year, or 21,266 kg of carbon per year, per 1 ha. This means that to sequester the carbon dioxide deficit of 5.15 billion tons of CO₂ (as carbon) per year, we would need 101,802,383 ha (1,018,023 km²).

Given the attainable high RGR of 0.5/day, a sensitivity analysis was performed to estimate the variation in the required area to achieve the required sequestration rate of atmospheric carbon per year based on the variation in the standing crop density, which ranges between 300 and 600 kg/ha. The largest estimate for the required area would thus be based on the lower end of the standing crop density of 300 kg/ha. The maximum required area would therefore be 1,527,036 km². On the other hand, the lowest possible estimate is calculated considering the maximum attainable standing crop density of 600 kg/ha. The minimum required area would therefore be 763,518 km².

Discussion

Significance of the Azolla ponds

To our knowledge, studies assessing *Azolla* cultivation for mitigating the annual global increase in CO₂ are absent. Such assessment is however possible to conduct given numerous

Fig. 2 Phases of *Azolla* growing cycle

Table 2 *Azolla* maximum attainable plant density

<i>Azolla</i> species	Maximum plant density (kg dry weight/ha) (Wagner 1997)	Maximum growth rates as dry weight (g/m ² /day)
<i>Azolla</i> spp.	NA	9–9.72; 4.5–5 (Brouwer et al., 2018; Speelman et al. 2009)
<i>A. filiculoides</i>	1700–5200	9.7 ± 0.4; 15.2 (Bocchi and Malgioglio, 2010; Brouwer et al. 2018)
<i>A. caroliniana</i>	3190	NA
<i>A. mexicana</i>	830–1100	NA
<i>A. pinnata</i>	640–2170	9 ± 6.2 (Brouwer et al., 2018)
<i>A. nilotica</i>	2610	8.1* (Wagner 1997)

*Calculated assuming an average *Azolla* moisture content of 91% (Bhaskaran and Kannapan, 2015).

studies that address various growth aspects of *Azolla* (Sathapathy and Chand 2010). To put things into perspective, the Amazon forest, which is the largest tropical rainforest, covering an area of 5.5 million km², and one of the major sinks for atmospheric carbon, is able to remove around 0.65 billion tons of atmospheric CO₂ (as carbon) per year (Brienen et al. 2015), which is around 12.6% of the annual CO₂ deficit assessed in this study. This is equivalent to CO₂ removal of 1,182 kg of CO₂ (as carbon) per 1 ha of the Amazon forest per year. The estimated required area for the *Azolla* ponds to sequester the CO₂ deficit would thus be on average around 18.5% of the Amazon forest area. Additionally, it is noteworthy to mention that given that *Azolla* ponds are able to remove 21,266 kg of CO₂ (as carbon) per 1 ha per year, their efficiency of CO₂ sequestration would be 18 times higher compared to the same area of the Amazon forest per year, which is especially important given the diminishing forest resources around the world and the decreasing capability of the oceans to absorb CO₂ due to global warming. The decision on the frequency of harvesting of *Azolla*, the amount of the harvested *Azolla* in relation to the size of the growing pond, and the amount of the *Azolla* to be left in the pond will ultimately depend on many factors that must be taken into consideration, mainly the specific characteristics of the *Azolla* species to be used. It is also important to note that it is useful to consider utilizing multiple *Azolla* species for setting up the proposed *Azolla* ponds. This can be attained by growing different *Azolla* species separately in different regions, assuming the specific environmental characteristics of the area where the ponds will be located. It can be also performed by mixing and growing multiple species together such that upon seasonal changes to the environmental conditions within the same pond, one of the species will be more tolerant. Therefore, in both cases, the combination allows for a year-round continuous production of *Azolla*.

Overall, the annual increase in the rate of CO₂ emissions has slowed down to a steady linear increase of around 2 ppm/year since the early 2000s, especially compared to the previous several decades which saw the rate increase constantly from around 0.5 to 2 ppm (Keenan et al. 2016).

A huge factor is the increased awareness towards green technologies, forest conservation, reforestation, and technological advances in fossil fuel-based energy applications, therefore generating much less CO₂ compared to previous generations (Miller and Spoolman 2019). Modeling of annual atmospheric CO₂ emissions is not a straightforward practice; however, *Azolla* sequestration ponds can allow for compensation of any unexpected increases. To better adapt the approach to the annual increase, one option would be simply growing *Azolla* to mitigate the impact of the previous year. By utilizing this approach, it will be much easier to exactly estimate the number of the sequestration ponds that must be employed. Therefore, the proposed *Azolla* CO₂ sequestration approach will contribute heavily to the capability of the biosphere in slowing the rate of CO₂ accumulation in the atmosphere.

Given that this study offers a baseline assessment of *Azolla* to be utilized as the sequester of CO₂, evaluation of the location of the ponds will therefore be of high importance as it heavily depends on the specific characteristics of each of the *Azolla* species. For example, different ponds might utilize different species based on the local growth conditions. Furthermore, assessment of the use of multiple *Azolla* species as a consortium within the same pond is also highly valued, as this might offer a faster growth compared to single species under a certain set of environmental conditions. This would open the door for future studies to assess the optimal locations for growing the *Azolla* ponds around the world. It is also important to note that future studies should also focus on the cost of construction and operation of the *Azolla* ponds as well as on assessing any potential emissions generated during the process of growing of *Azolla* that would allow for optimizing the design of the ponds more sustainably.

It is also important to note that changes in the atmospheric CO₂ levels correlate strongly with global warming and with climate change, and thus, this might have an impact on the carbon cycle through vegetation. This might require future modification of the proposed approach to accommodate any future limitations.

Potential impact on atmospheric CO₂ and global warming

One important consideration of the proposed *Azolla* sequestration approach is that offsetting the annual fossil carbon emissions in the atmosphere would lead to a gradual decrease in atmospheric CO₂ levels over the years. This is mainly because, with no additional carbon being added into the atmosphere, the carbon cycle will start shifting towards capturing of the atmospheric carbon, which would ultimately lead to lowering of its corresponding atmospheric concentration. An important consequence of such a decrease is the potential reduction in the average temperature of the Earth, which can be considered a direct positive impact of the *Azolla* sequestration setup on global warming. To assess this potential impact, a simple Earth Model simulation was performed to estimate the change in the atmospheric CO₂ concentration and the average Earth temperature over the next few decades. Simple Climate Model was utilized for this purpose (UCAR 2021). Two separate simulations were performed. Figure 3 shows the results of the simulation. The first simulation (Fig. 3a) shows the expected change in the atmospheric CO₂ concentration and the average Earth temperature assuming that the global fossil emissions will remain similar to the annual average of 9.4 billion tons of C per year over the past decade (Friedlingstein et al., 2020). The second simulation (Fig. 3b) shows the same output considering that the global fossil emissions were offset by the *Azolla* ponds, leading to zero net annual emissions of atmospheric CO₂.

The results show that if the fossil CO₂ emissions continued at the recent average (Fig. 3a), it would significantly add to the atmospheric CO₂ concentrations, continuing to increase from current 400 to reach 550 ppm by the year 2100. This is also accompanied by a continuous rise in the average temperature of the Earth, reaching an average of

16.04 °C by 2100. On the other hand, if the annual carbon emissions dropped to negligible levels upon application of the proposed *Azolla* ponds, we would not only see a substantial drop in the atmospheric CO₂ by 2100 to 366 ppm (levels last seen in 1998), but we will also expect a considerable drop in the average temperature of the Earth to 14.28 °C. Hence, the proposed *Azolla* sequestration approach has a significant potential to reduce the impacts of global greenhouse gas emissions and global warming.

Potential uses of the harvested biomass

It is important to note that the harvested *Azolla* biomass from the proposed *Azolla* ponds would have significant potential uses. The major method to effectively utilize *Azolla* for sequestration of CO₂ is locking the biomass away, thus permanently removing the CO₂ from the atmosphere. This could be achieved for example by burial of the grown biomass in deep geologic formations, which would provide an easy and energy efficient process of mitigating the impacts of the released fossil fuel carbon from anthropogenic activities (Sayre 2010; Zeng 2008). Another major use of the harvested biomass would be using it as a feed for various animal resources, therefore saving on the cost and environmental impacts of production of animal feeds. *Azolla* biomass can be adapted as an alternate and sustainable component incorporated into the feed formula or as a protein feed of a variety of animals. This includes livestock such as cattle, swine, and poultry, and even includes feeds utilized in fish farming (Alalade and Iyayi, 2006; Ara et al. 2015; Basak et al. 2002; Das et al. 2018; Kathirvelan et al. 2015; Mosha 2018; Oktavianawati et al. 2016; Pillai et al. 2002). It is important to note utilizing the biomass in certain ways, like as a feed or a biofuel, might ultimately lead to the release of the sequestered carbon back into the atmosphere. However, it is also important to note that such uses would indirectly

Fig. 3 Potential impact of the *Azolla* ponds on global warming and atmospheric CO₂ levels

contribute to long-term reduction in the atmospheric CO₂ due to lowered demand for fossil fuels. The rerelease of the captured carbon into the atmosphere by utilizing the biomass as a source of carbon means that a significant portion of fossil fuel-derived carbon will not be added to the atmosphere, thus reducing the annual addition of fossil carbon into the atmospheric reservoir of the carbon cycle.

Azolla biomass can be also used as a biofertilizer and as a soil amendment product to enhance the organic content of certain soils, therefore increasing the yield and quality of crops at reduced prices. This is especially important given the nitrogen content of the *Azolla* that is assimilated from the atmosphere, thus reducing the reliance on production and use of inorganic fertilizers that have significant environmental impacts (Miller and Spoolman 2019). For example, Yao et al. (2018) demonstrated the capability of *Azolla* as a biofertilizer for improving low efficiency in an intensive rice cropping system. Other authors also demonstrated the use of *Azolla* as a biofertilizer for enhanced production of a variety of crops and vegetables, which could help in moving towards sustainable agriculture (Cabbage et al. 2019; Hanafy et al. 2018; Kollah et al. 2016).

Finally, another important potential use for the harvested *Azolla* biomass could be the production of biofuels for bio-energy, which would not only decrease the demand for fossil fuels, but would also provide high energy fuels at a reduced cost compared to other types of biofuels (Roy et al. 2016).

Potential drawbacks

The current pause in the increase of growth rate of atmospheric CO₂ might suggest that the rate might become negative in the future, with less carbon being added each year compared to the previous years. It does not however imply that this is certain. This is mainly because any predicted decline in the rate is heavily dependent on future CO₂ emissions. Implementation of the proposed *Azolla*/CO₂ sequestration approach would be a major step, nonetheless, as it might be a trigger to the decline in the growth rate of atmospheric annual CO₂ accumulation. This might however have some drawbacks that should be taken into consideration. One major consideration is that *Azolla* can turn into an invasive weed in certain ecosystems, which is the reason behind several studies addressing control measures for *Azolla* (Farahpour-Haghani et al., 2019; Madeira et al. 2016; Mvandaba et al. 2019; Pinero-Rodríguez et al. 2019). To utilize the proposed large *Azolla* ponds, it might be hard to control the spread of *Azolla* macrophytes into nearby water bodies or its accidental transfer, which could provide the possibility for *Azolla* to dominate the indigenous ecosystem in the absence of natural consumers. Thus, strict planning and control measures should be assessed in order to minimize the potential invasion of

Azolla. This could be addressed by careful evaluation of the locations, nearby ecosystems, and even the utilized *Azolla* species. On the other hand, although *Azolla* can be very robust in terms of its growth characteristics, it can be highly fragile and intolerant to certain environmental factors (SI and Table 1). Sudden changes to environmental factors such as extreme seasonal variations will result in suppression of *Azolla* proliferation, decreasing thus the productivity of the plant (Arora 2003; Espinar et al. 2015; Katole et al. 2017; Sadeghi and Zarkami 2013). Furthermore, even when environmental conditions are optimal for *Azolla* growth, certain biological factors, such as the presence of certain insects like the weevil *Stenopelmus rufinasus* (Shaw et al. 2018), could mean significant hindrance to the growth of *Azolla*, and could lead to ultimate failure of the ponds. That is why one suggestion is to utilize different species of *Azolla* in different areas, and even to use a consortium of different species in the same ponds, which would increase the tolerance of the growing biomass to environmental factors, ultimately maintaining a high productivity and reducing inhibitory events. Additionally, it is important to note that for continuous growing of the biomass, although *Azolla* is not limited by nitrogen, it is important to provide sufficient amounts of limiting macronutrients such as phosphorus, potassium, calcium and magnesium, and even micronutrients like iron, molybdenum, manganese, zinc, and copper (Biswas et al. 2005; Kushari and Watanabe 1992; Serag et al. 2000; Singh et al. 2010).

It is noteworthy to mention that given the wide range of optimal environmental conditions of different *Azolla* species, it would be expected that the *Azolla* pond locations could be adapted to any area of the world, therefore not limiting the approach to indigenous tropical and subtropical areas. As a result, future studies are recommended to address evaluation of specific locations of the *Azolla* ponds in relation to the available environmental conditions and the potential *Azolla* species that would be used in that specific area.

Last but not least, it also important to mention that operating the *Azolla* ponds might result in some CO₂ emissions, which need to be also taken into consideration when operating the ponds to compensate for these emissions.

Conclusion

The Eocene *Azolla* bloom event that significantly contributed to the capturing of atmospheric CO₂, contributing to the cooling of the Earth, will always be a reminder of the capability of *Azolla* as a fast sequester of CO₂. This is especially important given that *Azolla* might achieve growth rates that are even faster than the current reported

ones if grown under specific conditions close to the Eocene's, namely the higher concentration of CO₂ in the atmosphere. Based on the assessment performed in this study, *Azolla* CO₂ sequestration ponds provide a novel natural method for CO₂ sequestration with much lower environmental impacts compared to current practices. Additionally, *Azolla* biomass would provide a usable product that can be utilized in a variety of fields, rendering the proposed approach also commercially valuable. This method will be hence an alternative green approach to mitigate the impacts of fossil fuels on ecosystems around the globe. Therefore, *Azolla* provides an important opportunity for mitigating the global carbon footprint while simultaneously being able to utilize it as a useful product.

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Declarations

Ethics approval and consent to participate Not applicable

Consent for publication Not applicable

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