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SUSTENTABILIDAD
ECOLOGÍA**

**ECOLOGÍA FISIOLÓGICA DE PLANTAS ATMOSFÉRICAS:
BIOMONITOREO, RESPUESTA AL DEPÓSITO DE NITRÓGENO E
IMPLICACIONES EN POLÍTICA PÚBLICA**

TESIS

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RESUMEN

El nitrógeno molecular es el componente más importante de la atmósfera, siendo el 78% de ella. Sin embargo, muchos ecosistemas alrededor del mundo están limitados por el nitrógeno debido a que el nitrógeno molecular no está disponible para la mayoría de organismos vivos. Esta tendencia ha cambiado desde la segunda mitad del siglo veinte, cuando el incremento en la población mundial ha conducido a un aumento en las tasas de emisión de nitrógeno reactivo a la atmósfera debido a la industria, automóviles, cultivos y ganadería. Estas actividades contribuyen con la ruptura del nitrógeno atmosférico y lo dejan disponible para los ecosistemas. Este proceso es conocido como depósito de Nitrógeno atmosférico y es considerado la tercera causa de pérdida de biodiversidad en el siglo veintiuno.

Esta tesis muestra los diferentes aspectos relacionados con las respuestas fisiológicas y metabólicas de plantas atmosféricas al ambiente, tal como cambios en la luz y humedad, así como al depósito de nitrógeno atmosférico. También, en esta tesis algunas nociones sobre política pública en relación a la contaminación atmosférica, monitoreo de la calidad del aire y salud pública son explorados con el fin de mostrar deficiencias y dar recomendaciones para un mejor conocimiento sobre este tema en México.

El uso de isótopos estables es una buena herramienta para rastrear procesos biogeoquímicos, en esta tesis fueron usados para determinar las fuentes potenciales de depósito atmosférico. En el capítulo dos, en el cual se revisa el uso de biomonitores para el depósito de nitrógeno, el uso de isótopos estables es ampliamente discutido. En el capítulo 3, isótopos estables de nitrógeno muestran las diferentes fuentes de nitrógeno para orquídeas y musgos creciendo en la ciudad y el campo. En el capítulo 4, isótopos estables de nitrógeno permitieron determinar la principal fuente de nitrógeno para orquídeas expuestas a diferentes dosis de depósito de nitrógeno simulado. En el capítulo 5, isótopos estables de nitrógeno muestran la distribución espacial de los contaminantes nitrogenados en el Valle de México por medio de diferentes biomonitores, también, los isótopos estables trajeron claridad sobre las tasas de depósito y saturación de los biomonitores estudiados.

ABSTRACT

Molecular nitrogen is the most important component of atmosphere, being the 78% of it. However, many ecosystems worldwide are limited by nitrogen, because molecular nitrogen in the atmosphere is not available for most of living organisms. This trend has been change since the second half of the twentieth century, when the increasing global population has led to increasing rates of emission of reactive nitrogen to the atmosphere because of industry, automobiles, crops and animal husbandry. These activities contribute to break the atmospheric nitrogen and bring it available for ecosystems. This process is known as nitrogen deposition, and it is considered the third cause of biodiversity loss in the twenty first century.

This dissertation shows different aspects related with the physiological and metabolic ecology responses of atmospheric plants to environment such as light and humidity changes, also to nitrogen deposition. Also, in this dissertation some notions about public policy in relation to atmospheric pollution, monitoring air quality and public health are explored to show deficiencies and raise recommendations for a better knowledge on this topic in Mexico.

The use of stable isotopes is a good tool for track biogeochemical processes, in this thesis are used by determine potential sources of deposition. In chapter two in which the use of biomonitors for nitrogen deposition is reviewed, the topic about stable isotopes is amply discussed. In chapter three, stable isotopes of nitrogen showed different source of nitrogen for orchids and mosses growing in the city and the country side. In chapter four, stable isotopes of nitrogen allowed to determine the main source of nitrogen for orchids exposed to different doses of simulated nitrogen deposition. In chapter five, stable isotopes of nitrogen showed the spatial distribution of nitrogenous pollutants in the Valley of Mexico by means of different biomonitors, also, stable isotopes brought a light about rates of deposition and saturation of biomonitors studied.

CAPÍTULO I:

INTRODUCCIÓN GENERAL

Artículo de divulgación:

¿Qué tanto es tantito? Una breve historia del nitrógeno en la era industrial

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¿QUÉ TANTO ES TANTITO? UNA BREVE HISTORIA DEL NITRÓGENO EN LA ERA INDUSTRIAL

Por: Edison Armando Díaz Álvarez, estudiante de Doctorado en el Posgrado en Ciencias Biológicas, en el Instituto de Investigaciones en Ecosistemas y Sustentabilidad (IIES)

EL NITRÓGENO ES UNO DE LOS ELEMENTOS QUE SE ENCUENTRA EN LA TABLA PERIÓDICA Y QUE, EN COMBINACIÓN CON CARBONO, OXÍGENO E HIDRÓGENO, CONSTITUYEN LOS BLOQUES BÁSICOS DE CONSTRUCCIÓN DE LA VIDA. El nitrógeno forma parte de compuestos orgánicos tales como el ADN, que es el encargado preservar y heredar la información genética. Este elemento también forma parte de los aminoácidos, que a su vez forman a las proteínas estructurales y a las enzimas que participan en el metabolismo celular. Además, el nitrógeno es uno de los fertilizantes más reconocidos para el crecimiento de las plantas, que son el primer eslabón de las redes tróficas.

El llamado nitrógeno molecular (N_2) constituye el 78% de la atmósfera terrestre, pero debido a su alta estabilidad, es difícil de romper y no está disponible para la mayoría de seres vivos. Este nitrógeno denominado "no reactivo" sólo puede ser utilizado (fijado) por algunos microorganismos especializados que pueden romperlo dejándolo disponible para las plantas. Un ejemplo son las bacterias del género *Rhizobium* comúnmente asociadas con plantas leguminosas. La ruptura del N_2 puede ocurrir durante las tormentas eléctricas, aunque también los incendios generan energía suficiente que contribuyen con la ruptura abiótica del N_2 . El producto de estas reacciones, conocido como nitrógeno reactivo (Nr), queda disponible para combinarse con otros elementos. Sin embargo, la cantidad de Nr producida de forma natural mediante estos procesos no es suficiente como para fertilizar los diferentes ecosistemas del planeta. Por ello, a pesar de la gran abundancia de este elemento en la atmósfera, la productividad de muchos ambientes terrestres está limitada por su escasez.

El nitrógeno se mueve a través del planeta en un proceso llamado ciclo de nitrógeno en el que diferentes reacciones químicas transfor-

man el nitrógeno desde su forma más estable en la atmósfera, hasta sus formas más complejas en la biosfera, donde forma parte de los seres vivos. En la época preindustrial más del 99% del nitrógeno atmosférico no estaba disponible para la gran mayoría de organismos y existía un equilibrio entre el nitrógeno que se fijaba y el nitrógeno que regresaba a la atmósfera. Sin embargo, diferentes actividades humanas contaminantes han llevado a que la disponibilidad de nitrógeno reactivo a nivel global se haya más que duplicado en el último siglo y con ello se haya desequilibrado del ciclo del nitrógeno.

El aporte de nitrógeno reactivo a la biosfera se denomina depósito de nitrógeno atmosférico, cuyo acelerado incremento tiene efectos adversos sobre la biodiversidad al punto de ser considerado como la tercera causa de pérdida de especies a nivel mundial, solo después del cambio de uso de suelo y del cambio climático. El depósito de nitrógeno es un fenó-


meno global que puede afectar sitios muy alejados, incluso si las emisiones no se producen allí, debido a que la contaminación puede ser transportada a través de la atmósfera por acción del viento.

Un pequeño aumento en el depósito de nitrógeno puede actuar como fertilizante al estimular el crecimiento vegetal y la absorción de carbono. Sin embargo, cuando este sobrepasa el umbral de tolerancia (que es diferente para cada especie y cada ecosistema) puede causar efectos negativos, tal como el desequilibrio de nutrientes, cambios en la composición de especies y acidificación del suelo y del agua, además de conducir a la saturación de diferentes ambientes. Debido a este fenómeno, los componentes bióticos y abióticos del ecosistema no pueden hacer uso de todo el nitrógeno que reciben, lo que puede llevar a tener efectos tóxicos.

Es importante implementar programas de monitoreo que permitan evaluar el estado de este fenómeno y sus efectos sobre los diferentes ecosistemas. Para ello se utilizan redes automatizadas de monitoreo atmosférico. Sin embargo, la operación de estaciones automatizadas de monitoreo atmosférico implica un alto costo. En México, un despliegue efectivo sólo ha sido posible en la Ciudad de México, Guadalajara y Monterrey. Mientras que en ciudades más pequeñas los datos de contaminación son insuficientes. Por lo que una alternativa para estimar la contaminación por nitrógeno tanto en las ciudades como en el campo es mediante el uso de biomonitores, cuyo contenido de nitrógeno y abundancia de isótopos estables en sus tejidos permiten caracterizar de este fenómeno.

Los estudios con organismos biomonitores del depósito de nitrógeno atmosférico, son cada vez más comunes debido a que permiten obtener resultados rápidos y a bajo costo en comparación con los métodos automatizados (estaciones de monitoreo). Dichos organismos dependen principalmente de los nutrientes que reciben de la atmósfera por lo que reflejan claramente la contaminación prevalente del lugar donde crecen. Ejemplos de organismos biomonitores son los líquenes, los musgos, las epífitas vasculares como bromélas y orquídeas. Estos biomonitores se caracterizan

por ser especies de amplia distribución geográfica y ecológica, por ser abundantes y que estén disponibles todo el año

Soy biólogo, candidato a doctor por el Posgrado en Ciencias Biológicas en el Instituto de Investigaciones en Ecosistemas y Sustentabilidad, bajo la supervisión del Dr. Erick de la Barrera. Mi investigación doctoral contempla el efecto de la contaminación atmosférica por nitrógeno en plantas tal como la orquídea nativa de México *Laelia speciosa*. Adicionalmente, con mi investigación busco determinar los biomonitores más adecuados para caracterizar la contaminación atmosférica por nitrógeno. 



LAELIA SPECIOSA, ORQUÍDEA EPÍFITA, ENDÉMICA DE MÉXICO, CRECIENDO SOBRE CORTEZA DE ENCINO (*QUERCUS DESERTÍCOLA*). ESTA ORQUÍDEA RESPONDE AL DEPÓSITO DE NITRÓGENO ATMOSFÉRICO AUMENTANDO EL CONTENIDO DE NITRÓGENO Y CAMBIANDO LA COMPOSICIÓN ISOTÓPICA DE SUS TEJIDOS. FOTO: EDISON ARMANDO DÍAZ ÁLVAREZ.

Objetivos

General

Por medio del estudio de diferentes plantas epifitas neotropicales y su respuesta al depósito de nitrógeno atmosférico, determinar la idoneidad de las mismas para ser utilizadas en estudios de biomonitorio en diferentes ambientes.

Específicos

- Dar un marco de referencia sobre el uso de biomonitores del depósito de nitrógeno atmosférico.
- Comparar la composición isotópica y el contenido de nitrógeno de orquídeas epífitas que creciendo en lugares contrastantes (campo y ciudad).
- Determinar la respuesta fisiológica de la orquídea *Laelia speciosa* a dosis crecientes de depósito de nitrógeno simulado.
- Determinar la distribución espacial de la magnitud del depósito de nitrógeno atmosférico por medio de diferentes organismos biomonitores.
- Analizar la normatividad en materia de contaminación atmosférica y evaluar el cumplimiento por parte de las autoridades locales, regionales y nacionales de los diferentes campos de aplicación de la NOM-156-SEMARNAT-2012.
- Determinar la respuesta fisiológica de la bromelia epífita *Tillandsia makoyana* a cambios medioambientales.

CAPÍTULO II

Artículo de revisión:

Biomonitors of atmospheric nitrogen deposition: potential uses and limitations

Biomonitoring of atmospheric nitrogen deposition: potential uses and limitations

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Atmospheric nitrogen deposition is the third largest cause of global biodiversity loss, with rates that have more than doubled over the past century. This is especially threatening for tropical regions where the deposition may soon exceed 25 kg of N ha⁻¹ year⁻¹, well above the threshold for physiological damage of 12–20 kg of N ha⁻¹ year⁻¹, depending on plant species and nitrogenous compound. It is thus urgent to monitor these regions where the most diverse biotas occur. However, most studies have been conducted in Europe, the USA and recently in China. This review presents the case for the potential use of biological organisms to monitor nitrogen deposition, with emphasis on tropical plants. We first present an overview of atmospheric chemistry and the nitrogen metabolism of potential biomonitors, followed by a framework for monitoring nitrogen deposition based on the simultaneous use of various functional groups. In particular, the tissue nitrogen content responds to the rate of deposition, especially for mosses, whose nitrogen content increases by 1‰ per kilogram of N ha⁻¹ year⁻¹. The isotopic signature, δ¹⁵N, is a useful indicator of the nitrogen source, as the slightly negative values (e.g. 5‰) of plants from natural environments can become very negative (–11.2‰) in sites with agricultural and husbandry activities, but very positive (13.3‰) in urban environments with high vehicular activity. Mosses are good biomonitors for wet deposition and atmospheric epiphytes for dry deposition. In turn, the nitrogen saturation of ecosystems can be monitored with trees whose isotopic values increase with saturation. Although given ecophysiological limitations of different organisms, particular studies should be conducted in each area of interest to determine the most suitable biomonitors. Overall, biomonitors can provide an integrative approach for characterizing nitrogen deposition in regions where the deployment of automated instruments or passive monitoring is not feasible or can be complementary.

Key words: Atmospheric monitoring, ecosystem saturation, environmental pollution, epiphytic plants, nitrogen content, stable isotopes

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Introduction

Nitrogen is one of the essential elements for life and the most abundant in the terrestrial atmosphere, 80% of which is composed of N₂ (Soderlund, 1976). Due to the high chemical

stability derived from its strong triple bond, this molecule can only be divided by processes involving large quantities of energy or through the action of specialized nitrogen-fixing microorganisms (Galloway *et al.*, 2003). For this reason, in the pre-industrial age, more than 99% of the atmospheric

nitrogen was unavailable for the great majority of organisms, which lack the enzyme nitrogenase required for fixing N_2 (White *et al.*, 2012). However, as a result of our growing human population and its associated demand for food and energy, the biologically available nitrogen has more than doubled in the atmosphere over the last century. Agriculture, industry and the use of automobiles are the main sources of a complex of chemical species known as reactive nitrogen (Nr), originated from the splitting of N_2 (Galloway *et al.*, 2008).

Such an increased deposition of atmospheric nitrogen has adverse effects on biodiversity. Indeed, this form of atmospheric pollution is considered to be the third largest threat to global biodiversity, following only changes in land use and climate (Sala *et al.*, 2000; Payne *et al.*, 2017). In particular, a deposition rate of 10 Kg of N $ha^{-1} year^{-1}$, which has already been recorded for some ecosystems, is sufficient to cause physiological damage in plants (Fenn *et al.*, 2003; Bobbink *et al.*, 2010; Simkin *et al.*, 2016; Payne *et al.*, 2017). Global projections of nitrogen deposition are especially threatening for tropical regions, where it could exceed 25 Kg of N $ha^{-1} year^{-1}$ during the present century (Galloway *et al.*, 2004, 2008; Phoenix *et al.*, 2006).

Implementation of monitoring programs that enable evaluation of the status of this phenomenon and its effects on different ecosystems is thus necessary, especially in the tropics where the most diverse biotas occur. However, the deployment and operational costs of automated air quality monitoring networks may exceed the financial capacity of developing countries. One economical alternative is the use of passive collectors, which are effective in tracking pollution over large areas. Another potential alternative for tracking the nitrogen that enters ecosystems is the use of biomonitor organisms, whose spontaneous occurrence in sites of interest allows an integrative assessment of nitrogen deposition even with a single collection event, as could be during an exploratory field campaign, or in extensive exploration efforts such as national forest surveys. A biomonitor, 'is an organism that contains information on the quantitative aspects of the quality of the environment' (Markert *et al.*, 2003). The particular species to be selected in each region of interest (i) should have an ample ecological and geographic distribution, (ii) should be abundant and available throughout the year and (iii) there should be a clear relationship between the variable of interest and the response of the bioindicators (Conti and Cecchetti, 2001).

This paper presents the case for the potential utility of direct measurements of the nitrogen content and isotopic signature of plant tissue for characterizing nitrogen deposition. We start by showing how reactive nitrogen is formed and released to the atmosphere through anthropic activities and discuss the isotopic variation of these chemical species. Next, we explore the advantages and disadvantages of using different types of biomonitors such as mosses and vascular plants, as well as their particular responses to the different forms of nitrogen.

Reactive species of nitrogen in the atmosphere

Agriculture releases reactive nitrogen through the volatilization and leaching of nitrogenated fertilizers (Fig. 1; Cameron *et al.*, 2013). In turn, husbandry contributes to such reactive nitrogen through volatilized ammonia gas (NH_3 ; Fowler *et al.*, 2013). Industrial activity and motor vehicles also release reactive nitrogen to the atmosphere through the combustion of fossil fuels and other processes that consume large quantities of energy, which break the triple bond of N_2 and form nitrogen oxides (NOx, i.e. NO and NO_2 ; Fig. 1; Galloway *et al.*, 2008).

Such nitrogen oxides and ammonia emitted to the atmosphere are subject to different chemical reactions that lead, for example, to the formation of water-dissolved compounds and gases (NO_3^- , NH_4^+ , HNO_3), and aerosols [$(NH_4)_2SO_4$ and NH_4NO_3] (Aneja, 2001). These compounds are subsequently transferred to the surface of the earth either as dry deposition, in which the atmospheric gases or aerosols or deposit by gravity, or as wet deposition, in which the nitrogen ions are deposited in fog, snow or precipitation (Fig. 1; Anderson and Downing, 2006; Decina *et al.*, 2017).

Isotopic composition of atmospheric reactive nitrogen

The isotopic values of reactive nitrogen in the atmosphere have a direct relationship with the source of emission (Box 1). For instance, biogenic emissions of the soil have very negative $\delta^{15}N$ values between -50‰ and -20‰ (Felix *et al.*, 2013; Felix and Elliott, 2014). Such an ample range of values for gaseous nitrogen species leads to differences in the $\delta^{15}N$ of the nitrogenous compounds that dissolve in atmospheric water. In particular, the isotopic values of NH_3 from volatilization of ammonia in the soil and animal wastes, tend to be low, as negative as -40‰ (Freyer, 1978, 1991; Kendall *et al.*, 2007; Felix *et al.*, 2014, 2017). In turn, the $\delta^{15}N$ for NO_3^- and for NH_4^+ range from -15‰ to 15‰ , where NO_3^- is usually less negative than NH_4^+ (Hoering, 1957; Heaton, 1990; Liu *et al.*, 2012a). In this respect, the negative values observed for the NH_4^+ are the result of the very negative NH_3 reacting in the atmosphere (Felix *et al.*, 2014, 2017). In addition, land use influences the $\delta^{15}N$ of NH_4^+ from wet deposition are less negative in rural areas, ranging from -7‰ to 1‰ , than in urban zones where they range from -16‰ to -5‰ (Ammann *et al.*, 1999; Stewart *et al.*, 2002; Xiao and Liu, 2002; Garten, 2006; Liu *et al.*, 2012a; Xiao *et al.*, 2012; Harmens *et al.*, 2014; Sheng *et al.*, 2014).

With respect to dry deposition, the isotopic signature of atmospheric NOx is the result of the synergy of various factors. For example, in gasoline and diesel vehicles, both the isotopic composition (positive or negative) and the nitrogen

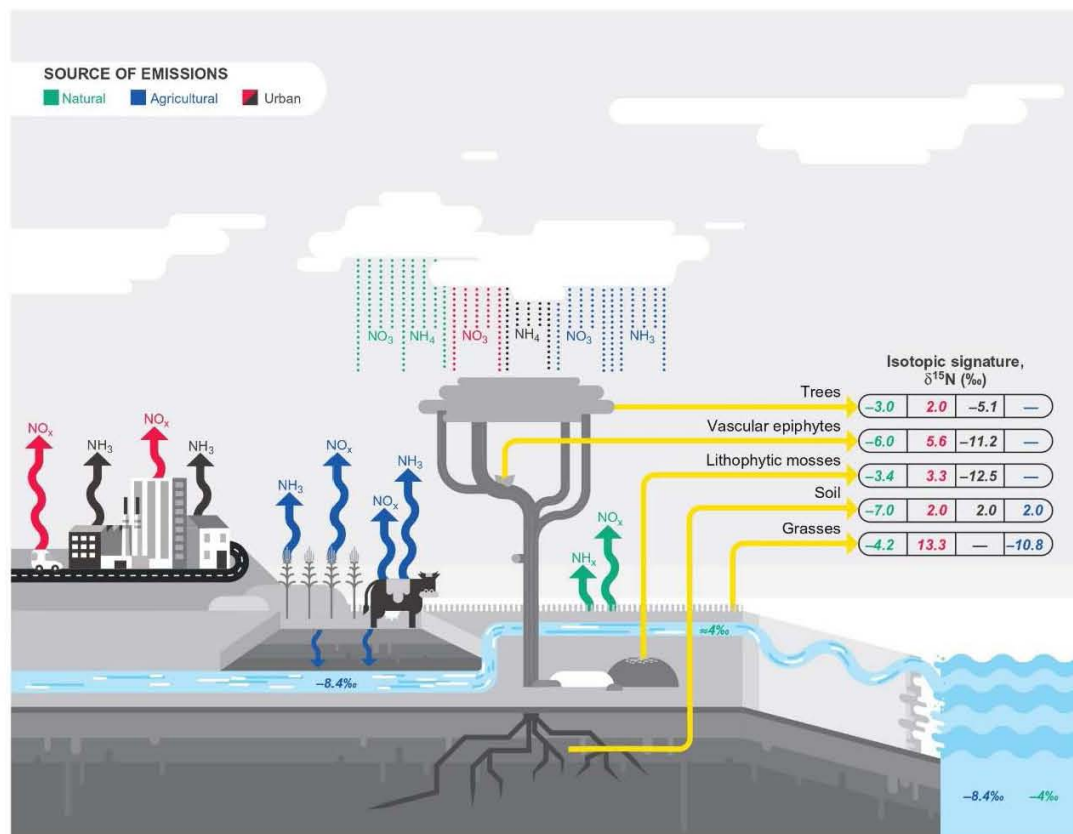


Figure 1: Sources and sinks of nitrogenous emissions. Isotopic composition of different biomonitors including trees, atmospheric plants, lithophytic mosses, grasses and the soil. Green lines and values indicate the isotopic composition of plants from natural environments and ensuing fluxes. Red indicates fluxes derived from exposure to NO_x emissions from urban environments, while black NH₃ from urban environments. Blue tracks the nitrogenous emissions from agriculture and husbandry.

concentration in the fuel interact with the amount of isotopic fractionation during combustion following the mixing of N₂ with O₂, which depends on the operation of the engine (Moore, 1977; Felix *et al.*, 2013). The burning of coal and trash can also result in an ample range of $\delta^{15}\text{N}$ values, depending on various factors, including the isotopic composition of the material burned, temperature, pressure and time of the reaction that influence fractionation (Box 1; Moore, 1977; Liu *et al.*, 2012a; Felix *et al.*, 2012, 2013; Felix and Elliott, 2014). For instance, the NO_x emitted by electrical energy plants (stationary source) through the combustion of coal has $\delta^{15}\text{N}$ values between 6‰ and 13‰ in South Africa and between 5‰ and 26‰ in China (Heaton, 1990; Li and Wang, 2008). Similarly, the $\delta^{15}\text{N}$ of the combustion of gasoline, diesel, natural gas and the incineration of trash in France yield values of 4.6–7.7‰ (Widory, 2007). In turn,

studies of roadside vehicular emissions have $\delta^{15}\text{N}$ of 3.7–15.0‰ (Moore, 1977). In contrast, the combustion of coal and fuel oil in the European country range from -7.5‰ to -5.3‰ (Widory, 2007). And the NO_x from the combustion of gasoline in vehicles (mobile source) in South Africa reach isotopic values of between -13‰ and -2‰ (Widory, 2007).

A framework for biomonitoring atmospheric nitrogen deposition

The use of biomonitors can provide an integrative assessment of ecosystem responses to nitrogenous pollution with consideration of the physiological, ecological and atmospheric conditions of the region of interest (Fig. 1; Sutton

Box 1: Stable isotopes and the δ notation

Isotopes are atoms of an element that have the same number of protons and electrons, but a different number of neutrons; i.e. they are of different atomic mass. Of the known elements, there are at least 300 stable isotopes. Some elements, such as tin, have up to ten, while 21 elements are known to only have one isotope (Sulzman, 2007).

For the case of nitrogen, there are two stable isotopes. ^{14}N is the most common and the lightest, with an abundance on Earth of 99.63%. In turn, the heaviest isotope is ^{15}N , with a terrestrial abundance of a mere 0.37% (Rosman and Taylor, 1997; Sulzman, 2007). A stable isotope is one that remains energetically stable over time; i.e. it neither emits energy nor decays, as it occurs with radioactive isotopes that gradually mutate towards a more stable state. The better known is the radioactive isotope of carbon, ^{14}C , which is widely used in archaeological studies (Sulzman, 2007).

Differences in the isotopic composition of some materials are so small that they are reported in parts per thousand (‰), relative to an international standard. The standard used for the isotopic analyses of nitrogen is the N_2 of the air. The isotopic abundance of a material is determined using the following formula:

$$\delta^{15}\text{N}(\text{‰}) = (R_{\text{sample}}/R_{\text{standard}} - 1) \times 1000,$$

where $\delta^{15}\text{N}$ is the isotopic proportion of the sample relative to the standard, R is the proportion between the heavy isotope and the light isotope, so that R_{sample} is the proportion in the sample and R_{standard} is the proportion in the standard (Evans, 2001).

In chemical reactions, the differences in the $\delta^{15}\text{N}$ of the substrate and the product result from a process known as isotopic fractionation through which the lighter isotope is favoured over its heavier counterpart. This process is described by (Δ)

$$\Delta = \delta^{15}\text{N}_s - \delta^{15}\text{N}_p,$$

where $\delta^{15}\text{N}_s$ is the isotopic composition of the substrate and $\delta^{15}\text{N}_p$ is the isotopic composition of the product (Evans, 2001). One tissue will be more enriched than another when it has a greater proportion of ^{15}N , and depleted in the opposite case. For the case of biological reactions, accumulated fractionation is known as isotopic discrimination.

Almost all chemical processes are subject to some degree of isotopic fractionation, in consequence relative abundances of an isotope can reveal the nature of the process from which it comes. Biological organisms are not the exception, all their metabolic reactions reveal their interaction with the environment, allowing track biogeochemical processes. In this case, stable isotopes, particularly of nitrogen, become an excellent integrative tool for understand the organism-environment interactions.

et al., 2004; Harmens *et al.*, 2014; Pinho *et al.*, 2017). Species composition and the physiological responses of biomonitor species following experimental manipulations have been amply utilized (Bobbink *et al.*, 2010; Ochoa-Hueso *et al.*, 2011; Lu *et al.*, 2012; Jones *et al.*, 2014). Here, we propose that a better approach to biomonitoring of nitrogen deposition is the determination of total nitrogen content and $\delta^{15}\text{N}$ from plant tissue, which can help characterize both the rate of deposition and the source of the nitrogenous pollution (Sutton *et al.*, 2004; White *et al.*, 2012; Díaz-Álvarez and de la Barrera, 2017). Indeed, various biogeochemical and physiological processes, as well as the determination of nitrogen sources, have been studied through measurements of the isotopic values of soil and plants, including for trees, herbaceous plants, mosses and vascular epiphytes (Emmett *et al.*, 1998; Stewart *et al.*, 2002; Wang and Pataki, 2011; Craine *et al.*, 2015; Díaz-Álvarez *et al.*, 2016; Felix *et al.*, 2016). In this case, the simultaneous consideration of an ensemble of biomonitors of different functional groups is necessary.

The total nitrogen content indicates the rate of nitrogen deposition

The total nitrogen content of biomonitors can help estimate the rate of atmospheric deposition in an ecosystem. In this case, epiphytic and lithophytic mosses are the best potential biomonitors because their tissue nitrogen content is determined by the prevailing atmospheric deposition. Mosses growing on the forest floor are also suitable biomonitors but to a lesser extent, given that the soil can contribute up to 37% of their tissue nitrogen content (Liu *et al.*, 2012a). Estimation of atmospheric deposition is thus possible from the nitrogen content of tissues, which increases by *ca.* 1% (dry weight) for each 10 Kg N ha⁻¹ year⁻¹ of deposition (Pitcairn *et al.*, 1998; Liu *et al.*, 2008c). This can be observed in natural areas of Europe, where the nitrogen content of mosses ranges between 0.5% and 0.7% and can double in polluted sites (Harmens *et al.*, 2011). However, the nitrogen content of mosses only increases linearly up to a threshold of 20 Kg N ha⁻¹ year⁻¹, after which it decreases progressively

(Pitcairn *et al.*, 2006; Shi *et al.*, 2017). Moreover, when the main form of nitrogen in deposition is NH_4^+ such a saturation is reached when this ion exceeds only $12 \text{ Kg N ha}^{-1} \text{ year}^{-1}$ (Pitcairn *et al.*, 1998; Wiedermann *et al.*, 2009). For instance, the nitrogen content of mosses decreases along pollution gradients in China, from 3.0% to 0.9% in urban areas and from 2.3% to 1.6% as pollution increases in rural areas (Liu *et al.*, 2008a,b; Xiao *et al.*, 2010). Given that the inherent nitrogen content of mosses varies amply among species, ranging from 0.1% to 0.5% for different species of pleurocarpus mosses (Pitcairn *et al.*, 1998; Wiedermann *et al.*, 2009; Harmens *et al.*, 2014), it is important to determine dose-response curves for the particular candidate biomonitors in each region of interest.

An important environmental factor that influences the relationship between nitrogen content of the mosses is precipitation. Indeed, the nitrogen content is better correlated with the rate of nitrogen deposition when the annual precipitation is above 1000 mm (Zechmeister *et al.*, 2008). The type of atmospheric deposition (wet or dry) also influences the nitrogen content of mosses. While wet deposition can cause a 0.01% increase in nitrogen content, dry deposition can lead to an increase of nitrogen content between 0.04% and 0.07% for each $1 \text{ Kg N ha}^{-1} \text{ year}^{-1}$, reaching up to 4% in sites with high rates of dry deposition of ammonia, but just up to 1.6% in sites with wet deposition (Hicks *et al.*, 2000; Solga *et al.*, 2005; Pitcairn *et al.*, 2006; Liu *et al.*, 2013a; Harmens *et al.*, 2014).

Vascular plants can also be utilized as biomonitors of the rate of nitrogen deposition, although care must be taken in their consideration as their responses are not linear. For example, the nitrogen content of the epiphytic orchid *Laelia speciosa* (Kunth) Schltr., 1914, amounts to 1.2% (dry mass) under a deposition of $10 \text{ Kg N ha}^{-1} \text{ year}^{-1}$, but $80 \text{ Kg N ha}^{-1} \text{ year}^{-1}$ are required to double the nitrogen content (Díaz-Álvarez *et al.*, 2015). This response has also been observed for seedlings of the tree species *Cryptomeria japonica* (Thunb. Ex L.f) and *Pinus densiflora* (Siebold & Zucc) and for adult individuals of *Pinus resinosa* Aiton. and *Schima superba* (Reinw. ex Blume) (Nakaji *et al.*, 2001; Zhang *et al.*, 2013). In this respect, an increased nitrogen availability often leads to the development of new tissue in vascular plants, rather than to increased levels in the existing cells, thus diluting what otherwise could amount to luxury nitrogen (Taiz and Zeiger, 2002).

Vascular plants can be an excellent complement to mosses for biomonitoring nitrogen deposition. Vascular plants prevail in environments that can be extreme for mosses to prosper, such is the case for urban heat island and arid regions. Additionally, given that vascular plants conform most of the plant cover, they are ideal for using other technologies such as remote sensing which can provide information about biomass and chlorophyll content variations as a result of alterations on atmospheric deposition (Schmidtlein *et al.*, 2012).

The isotopic composition discerns among natural, agricultural and urban nitrogen sources

The $\delta^{15}\text{N}$ of plants depends on multiple factors, including mycorrhizal associations, form of nitrogen used, soil depth accessed, but most importantly atmospheric sources (Fig. 1; Table 1). Indeed, epiphytic and lithophytic plants growing in natural sites without exposure to nitrogenous pollution have $\delta^{15}\text{N}$ that are negative but very close to zero (Wania *et al.*, 2002). In contrast, volatilization and leaching from agricultural and husbandry activities alters the isotopic composition of the vegetation, making it very negative (Craine *et al.*, 2015).

In urban environments the isotopic composition of plants can be positive or negative, depending on the dominant species of reactive nitrogen in the atmosphere (Fig. 1). For instance, in cities where the predominant nitrogen species are gaseous NH_3 and rain bound NH_4^+ , the $\delta^{15}\text{N}$ tend to be very negative (Xiao *et al.*, 2010; Liu *et al.*, 2012b; Felix *et al.*, 2013, 2017). This has been documented for urban mosses in China (Liu *et al.*, 2008a,b,c, 2012a,b, 2013b; Xiao *et al.*, 2010) and for urban plants in the vicinity of a fertilizer factory in Brazil, whose $\delta^{15}\text{N}$ reaches -41% (Stewart *et al.* 2002; Heaton *et al.*, 2004).

In contrast, the isotopic signature of urban plants from various functional types is positive when NO_x is the main source of nitrogen (Fig. 1). This has been documented for different mosses, including *Bryum argenteum* (Hedw) and *Grimmia pulvinata* (Hedw) in London and *Braunia* sp. and *Grimmia* sp. in Mexico City (Pearson *et al.*, 2000; Díaz-Álvarez and de la Barrera, 2017). Such positive values of $\delta^{15}\text{N}$ have also been measured for grasses in the megalopolis of Los Angeles (Wang and Pataki, 2009). The vicinity of roads, where NO_x from motor vehicles are emitted, can also determine the isotopic signature in otherwise natural environments, as positive $\delta^{15}\text{N}$ have been measured for the needles of the conifers *Picea abies* (L.) H. Karst. from Norwegian forests (Ammann *et al.*, 1999) and *Pinus edulis* (Engelm) within the Grand Canyon National Park in the USA (Kenkel *et al.*, 2016). A similar response to NO_x from motor vehicles has been documented for vascular epiphytes from west-central Mexico such as the orchid *Laelia speciosa* and the bromeliad *Tillandsia recurvata* (L.) (Díaz-Álvarez *et al.*, 2016; Díaz-Álvarez and de la Barrera, 2017).

A group with special potential for biomonitoring nitrogenous pollution in tropical regions is the so called atmospheric plants, a group that includes those epiphytes and lithophytes whose nutrition relies almost exclusive on deposited nutrients. Indeed, given their cosmopolitan distribution atmospheric mosses are widely utilized biomonitors (Markert *et al.*, 2003). Moreover, they are particularly adequate for tropical regions, where they reach their maximum diversity (Cárdenas and

Table 1: Isotopic values for different plants from contrasting environments

Life form	Species	Rural $\delta^{15}\text{N}$	Urban $\delta^{15}\text{N}$	Reference
Moss	<i>Braunia</i> sp.	-3.4‰	3.3‰	Díaz-Álvarez <i>et al.</i> (2016)
Mosses	8 species	-12‰	6.0‰	Pearson <i>et al.</i> (2000)
Mosses	4 species	-1.4‰	-12.5‰	Liu <i>et al.</i> (2008a)
Moss	<i>Haplocladium microphyllum</i> (Hedw)	-1.3‰	-6.5‰	Liu <i>et al.</i> (2008b)
Mosses	4 species	-7.9‰	-3.9‰	Liu <i>et al.</i> (2012a)
Annual C ₃ grasses	4 species	-4.2‰	13.3‰	Wang and Pataki (2009)
Herb	<i>Impatiens</i> sp.	-1.2‰	-6.1‰	Stewart <i>et al.</i> (2002)
Herb	<i>Calluna vulgaris</i> (L.)	-8.6‰	0.2‰	Power and Collins (2010)
Vascular epiphytes	8 species	-3.0‰	-10.9‰	Stewart <i>et al.</i> (2002)
Epiphytic bromeliad	<i>Tillandsia recurvata</i>	-6.0‰	3.0‰	Zambrano <i>et al.</i> (2009)
Epiphytic bromeliad	<i>Tillandsia usneoides</i>	-11.2‰	-2.2‰	Felix <i>et al.</i> (2016)
Epiphytic orchid	<i>Laelia speciosa</i>	-3.1‰	5.6‰	Díaz-Álvarez <i>et al.</i> (2016)
Tree	<i>Eriotheca</i> sp.	-1.6‰	-5.1‰	Stewart <i>et al.</i> (2002)
Tree	<i>Picea abies</i>	-3.0‰	2.0‰	Ammann <i>et al.</i> (1999)

Delgadillo, 2009). However, mosses depend on the availability of water for sustaining metabolic activity, thus their monitoring potential is limited to the rainy season. In contrast, succulent epiphytes, especially those with CAM photosynthesis, can be metabolically active throughout the year, thus providing a continuous record of atmospheric deposition regardless of seasonal weather variations (Ammann *et al.*, 1999; Andrade *et al.*, 2007; Zotz *et al.*, 2010). Orchids, for instance, can be found in multiple ecosystems throughout the tropics from sea level up to the subalpine forest above 3500 m (Ernshaw *et al.*, 1987). Atmospheric plants thus, allow a relatively accurate determination of both the source and the magnitude of atmospheric deposition with a very low or null isotopic discrimination given a direct water flux into the cells of mosses (Liu *et al.*, 2012a). In this case, nitrogen is subject to foliar uptake, either by direct influx of gaseous or aqueous nitrogen, i.e. NO, NO₂, NH₃ and HNO₃, directly from the atmosphere during gas exchange or when nitrogen particles are deposited on the plant and dissolved in rain or fog allowing the absorption of the ions NO₃⁻ and NH₄⁺ (Hietz *et al.*, 2002; Vallano and Sparks, 2008; Padgett *et al.*, 2009).

Although, atmospheric plants can pick up the isotopic signal of atmospheric deposition, care must be taken when, developing atmospheric biomonitors given the occasional presence of functional roots can obscure the isotopic signal measured from plant tissues (Hietz *et al.*, 2002; Reyes-García and Griffiths, 2009; Liu *et al.*, 2012a). Indeed, epiphytic plants that root in the canopy soil tend to be enriched in ¹⁵N compared with those that grow on thinner branches, where no substrate accumulation occurs, because the decomposition of the accumulated organic matter produces nitrogenous compounds with $\delta^{15}\text{N}$ close to zero (Wania *et al.*,

2002). Such a canopy soil originated from debris of the phorophyte is depleted in ¹⁵N relative to the forest soil which tend to accumulate ¹⁵N as the volatilization and biological uptake of the lighter isotope is favoured (Wania *et al.*, 2002; Liu *et al.*, 2012a; Craine *et al.*, 2015).

Trees indicate ecosystem nitrogen saturation

The $\delta^{15}\text{N}$ of trees is a good indicator of the state of saturation of atmospheric nitrogen in an ecosystem. The leaves and roots of the trees of N-saturated ecosystems tend to have positive $\delta^{15}\text{N}$, because saturation increases soil nitrification, a process that involves high rates of isotopic fractionation (Fig. 1; Box 1). In general, plants of ecosystems exposed to low rates of atmospheric deposition tend to present $\delta^{15}\text{N}$ that are negative but close to zero (Craine *et al.*, 2015). However, saturation leads to increased rates of nitrate leaching, which in turn causes saturated soils to become enriched with ¹⁵N, thus their $\delta^{15}\text{N}$ can become positive. Saturation also makes the relation between foliar $\delta^{15}\text{N}$ and nitrification closer than that between foliar $\delta^{15}\text{N}$ and the $\delta^{15}\text{N}$ of the nitrogen deposition (Ollinger *et al.*, 2002; Pardo *et al.*, 2006; Emmett, 2007). The opposite occurs for translocated nitrogen as a series of isotopic fractionations occurs as it moves from the roots to the branches to the leaves, because a series of enzymes such as nitrate reductase, nitrite reductase and glutamine synthetase are involved in nitrogen transformation, and each one has its own amount of discrimination (Evans, 2001).

Associations with mycorrhizal fungi also influence the $\delta^{15}\text{N}$ of the plants, and trees in particular, having the

potential to alter both the nitrogen relations of the plants and the isotopic signature of the assimilated nitrogen (Craine *et al.*, 2009, 2015). Under natural conditions (lower rates of atmospheric deposition), mycorrhizae supply their hosts with nitrogen that is depleted in ^{15}N (Emmett *et al.*, 1998). However, saturation can induce species turnover within the mycorrhizal community, from species with high amounts of isotopic discrimination against ^{15}N to species with low discrimination, contributing to the isotopic enrichment of the plants and the homogenization of the isotopic signature of the ecosystem (Emmett *et al.*, 1998; Craine *et al.*, 2009; Sheng *et al.*, 2014).

Metabolic limitations of biomonitors

Biomonitoring can become useful tools for detecting nitrogenous pollution over wide areas of terrestrial ecosystems. However, organismal responses are constrained by enzymatic processes. For brevity, this discussion is restricted to the metabolic limitations of mosses, which assimilate NH_4^+ to a greater extent when supplied simultaneously with NO_3^- . Likewise, these organisms preferentially assimilate organic compounds such as amino acids. For example, under simultaneous application of glycine with NH_4^+ and NO_3^- , assimilation of this amino acid is up to two times greater than that of the nitrate (Wanek and Pörtl, 2008; Wiedermann *et al.*, 2009). The main reason for this is the high energetic cost of assimilation of NO_3^- , which requires two consecutive reactions. In the first, NO_3^- is reduced to NO_2^- by the enzyme nitrate reductase, consuming two electrons in the process. In the second, NO_2^- is reduced to NH_4^+ by nitrite reductase, using six electrons (Heldt and Piechulla, 2011).

Nitrate reductase can be inhibited by assimilation of NH_4^+ from atmospheric deposition when the ratio between NH_4^+ and NO_3^- is high (Liu *et al.*, 2012a). Furthermore, high rates of atmospheric deposition can reduce or even completely inhibit nitrate reductase activity, whether it is due to the strong relationship between NH_4^+ and NO_3^- , or to the increased concentration of NO_3^- in the deposition of nitrogen. Indeed, while certain concentrations of NO_3^- are necessary to stimulate nitrate reductase synthesis and activity, an excessive amount of the ion exerts a negative feedback on the enzyme (Heldt and Piechulla, 2011). For this reason, when atmospheric deposition reaches $10 \text{ Kg N ha}^{-1} \text{ year}^{-1}$, a significant reduction is observed in the assimilation of NO_3^- and, on exceeding $30 \text{ Kg N ha}^{-1} \text{ year}^{-1}$, the nitrate reductase in the mosses is totally suppressed (Gordon *et al.*, 2002; Forsum *et al.*, 2006; Liu *et al.*, 2012a,b). High concentrations of atmospheric NO_x (greater than 63 nL L^{-1}) cause suppression of nitrate reductase in mosses of different anthropic environments. Exposure to NO causes nitrate reductase activity to decrease within 24 h, while exposure to NO_2 causes such an activity reduction over 21 days leading to the complete loss of inducibility of nitrate reductase even when NO_3^- is available (Morgan *et al.*, 1992; Forsum *et al.*, 2006; Liu *et al.*, 2012a,b).

Reduced assimilation of nitrate forces the mosses to assimilate other nitrogenated compounds in the atmospheric deposition, the different isotopic values of which are presented in Table 1. As a consequence, inhibition of nitrate reductase can cause variation in the isotopic values of moss tissues and can make determination of the source of the nitrogen observed in the tissue differ from the true source by up to 21% (Liu *et al.*, 2012a,b).

Thus, inhibition of nitrate reductase can cause a discrepancy between the nitrogen content of the mosses and the rate of atmospheric deposition on the site they inhabit. This can occur because nitrate that is deposited on the mosses can be partially assimilated or may not be assimilated at all. This will depend on the degree of inhibition of nitrate reductase. Consequently, part of the deposition (which contains the nitrate) will not be accurately recorded. In this case, estimation of atmospheric deposition could be more accurate in mosses when the ratio between NH_4^+ and NO_3^- is higher than in deposition with low NH_4^+ and NO_3^- ratios (Liu *et al.*, 2012a). It has been observed that the nitrogen content of mosses is lower under wet than under dry deposition (Pitcairn *et al.*, 2006; Liu *et al.* 2012a,b). Because mosses lack an epidermal cuticle, the inhibition of nitrate reductase may contribute to the leaching of a fraction of the deposited nitrate instead of being stored in the tissues of these organisms. In contrast, the leaching of unassimilated nitrogen during excessive wet deposition is greatly prevented by the cuticle for vascular plants (Pitcairn *et al.*, 2006; Liu *et al.* 2012a,b).

Monitoring nitrogen deposition by means of different organisms can be a useful tool for estimating the rate of nitrogen deposition in many regions. However, caution must be taken because the inhibition of the nitrate reductase above a species-specific threshold can lead to underestimations of actual deposition rate.

Perspectives

The nitrogen content and isotopic values of biomonitors can be suitable to inform environmental policy design for reducing the emissions of nitrogenous compounds, thus contributing to the mitigation of the adverse effects that atmospheric nitrogen deposition may have on priority ecosystems. Mosses can be especially useful because their nitrogen content responds directly to the rate of atmospheric deposition and their isotopic signature to the source. This is true up to certain deposition rate above which N accumulation decreases as a result of nitrate reductase inhibition. With the simultaneous use of different types of biomonitors, a multidimensional evaluation can be carried out regarding the state of ecosystems in the tropics. This could involve biomonitors that indicate the state of saturation, such as trees and shrubs, and those that indicate the source, such as vascular epiphytes with which it is possible to estimate the rate of atmospheric deposition using mosses. Further research

should consider the ‘calibration’ and development of potential biomonitors suitable for each region of interest. For the case of tropical regions, atmospheric plants may prove particularly adequate. In any case, caution must be exercised given that biomonitors cannot provide the exact magnitude of atmospheric deposition, but a semiquantitative approximation, including characterizing the nitrogen source. In this case, the simultaneous use of an ensemble of various species can be of great utility in identifying areas subject to pollution by atmospheric nitrogen, especially in regions where nitrogen saturation has not occurred.

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CAPÍTULO III

Artículo:

A $\delta^{15}\text{N}$ assessment of nitrogen deposition for the endangered Epiphytic orchid *Laelia speciosa* from a city and an oak forest in Mexico

A $\delta^{15}\text{N}$ assessment of nitrogen deposition for the endangered epiphytic orchid *Laelia speciosa* from a city and an oak forest in Mexico

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Abstract Atmospheric nitrogen deposition poses a major threat to global biodiversity. Tropical epiphytic plants are especially at risk given their reliance on atmospheric sources of nutrients. The leaf, pseudobulb, and root carbon and nitrogen content, C:N ratio, as well as the nitrogen isotopic composition were studied for individuals of *Laelia speciosa* from a city and from an oak forest in Mexico. The nitrogen content of leaves was similar between the city and the oak forest, reaching $1.3 \pm 0.2\%$ (dry mass). The $\delta^{15}\text{N}$ of leaves, pseudobulbs, and roots reached $5.6 \pm 0.2\text{‰}$ in the city, values found in sites exposed to industrial and vehicular activities. The $\delta^{15}\text{N}$ for plant from the oak forest amounted to $-3.1 \pm 0.3\text{‰}$, which is similar to values measured from sites with low industrial activities. Some orchids such as *Laelia speciosa* produce a single pseudobulb per year, i.e., a water and nutrient storage organ, so the interannual nitrogen deposition was studied by considering the ten most recent pseudobulbs for plants from either site formed between 2003 and 2012. The C:N ratio of the ten most recent pseudobulbs from the oak forest, as well as that of the pseudobulbs formed before 2010 for plants in the city were indistinguishable from each other, averaging 132.4 ± 6.5 , while it was lower for the two most recent

pseudobulbs in the city. The $\delta^{15}\text{N}$ values of pseudobulbs from the oak forest averaged $-4.4 \pm 0.1\text{‰}$ for the entire series. The $\delta^{15}\text{N}$ ranged from $0.1 \pm 1.6\text{‰}$ for the oldest pseudobulb to $4.7 \pm 0.2\text{‰}$ for the pseudobulb formed in the city from 2008 onwards. Isotopic analysis and the C:N ratio for *L. speciosa* revealed that rates of nitrogen deposition were higher in the city than in the forest. The $\delta^{15}\text{N}$ values of series of pseudobulbs showed that it is possible to track nitrogen deposition over multiple years.

Keywords Atmospheric pollution · Conservation physiology · Global change · Industrial activities · Neo-Volcanic axis

Introduction

Atmospheric nitrogen deposition has more than doubled since the mid-20th century as a result of an increased release of reactive nitrogen species from industrial and agricultural origin (Galloway et al. 2008; Phoenix et al. 2006). This important component of global change has driven a loss of biodiversity, which has been widely documented in Europe, the USA, and China (Bobbink et al. 2010; Xue-Yan et al. 2008). However, studies are scant for megadiverse countries like Mexico, which tend to have developing economies and ongoing industrialization processes, making it urgent to determine the effects of nitrogen deposition on their biodiversities (Austin et al. 2013). In this respect, an interest on tracing sources of deposition has increased and it can be studied by means of the isotopic composition of the vegetation.

Atmospheric nitrogen deposition confers a distinctive isotopic signature to vegetation. The isotopic composition of the reactive nitrogen species (NO_x and NH_x) that

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result from the atmospheric N_2 rupture can be either positive or negative (Heaton 1990; Moore 1977). In particular, the NOx from electric plants, coal, gasoline, diesel, and trash burning has $\delta^{15}N$ between +3.7 and +26 ‰ (Widory 2007). However, these isotopic values can also be negative, ranging from –13 to –2 ‰ (Redling et al. 2013). Whether the values are negative or positive will depend on the type of material burnt, the nitrogen in the fuel, and the various isotopic fractionations associated with the thermal production of NOx, when the fuel goes through the engine and is mixed with air O_2 and N_2 during the combustion (Felix and Elliot 2014; Felix et al. 2013; Liu et al. 2012). The $\delta^{15}N$ of biogenic emissions (NHx) of soils in rural areas are negative and can fluctuate between –50 and –20 ‰, including, organic wastes of animals and volatilization of nitrogen from agriculture (Felix et al. 2012; Hoering 1957; Li and Wang 2008; Felix et al. 2014). Once emitted the NOx or the NHx react with various compounds in the atmosphere, such reactions involve isotopic fractionations, that result in compounds such as NO_3^- and NH_4^+ , with different $\delta^{15}N$ values in rain ranging from –15 to +15 ‰, the NH_4^+ is more negative than the NO_3^- (Elliot et al. 2009; Heaton 1990; Heaton et al. 2004; Xiao and Liu 2002; Xiao et al. 2012). These isotopic values directly affect the ^{15}N composition of plants of different sites. In particular, plants exposed to industrial and vehicular emissions tend to be ^{15}N -enriched. For example, the $\delta^{15}N$ of mosses growing close to busy urban roads range between –1 and 6 ‰, while plants from less polluted areas tend to be ^{15}N depleted as is the case for mosses in rural areas, whose $\delta^{15}N$ values range from –2 to –12 ‰ (Ammann et al. 1999; Liu et al. 2007; Pearson et al. 2000; Power and Collins 2010; Stewart et al. 2002). However, in cities where the industrial activities release NHx, the $\delta^{15}N$ of plants are more negative than in the field (Liu et al. 2008; Stewart et al. 2002; Xiao et al. 2010).

A lifeform of particular interest for studying the effects of deposition is that of epiphytes, which account for up to 50 % of vascular plant species in some tropical ecosystems and are particularly susceptible to atmospheric nitrogen deposition, given their reliance on atmospheric sources of nutrients (Mondragón et al. 2015). These plants, whose roots have no contact with the forest soil, where the nitrogen fractionation takes place, reflect the $\delta^{15}N$ of the prevalent atmospheric nitrogen deposition (Delwiche and Steyn 1970; Hietz et al. 1999; Stewart et al. 2002; Zotz et al. 2010).

Laelia speciosa (Kunth) Schltr (Orchidaceae) is an endangered endemic epiphytic orchid from central Mexico with significant cultural importance (Halbinger and Soto-Arenas 1997). Vast numbers of individuals are illegally extracted and sold in streets and markets during May and June owing to their colorful flowers. This has caused a significant reduction of natural populations that has led to its

special protection status by the environmental authority (Mondragón et al. 2015; SEMARNAT 2010; Soto-Arenas and Solano-Gómez 2007). Moreover, oak forests, the natural habitat for this orchid, are highly threatened by changes in land use and climate (Gudiño et al. 2015; Rehfeldt et al. 2012; Soto-Arenas and Solano-Gómez 2007; Villers-Ruiz and Trejo-Vázquez 2000).

Under increasing rates of nitrogen deposition of up to 20 kg N ha⁻¹ year⁻¹, the carbon and nitrogen content increase and the C:N ratio decreases in epiphytes responding to increased photosynthetic rates (Díaz-Álvarez et al. 2015). But higher doses of nitrogen can produce noxious physiological effects on epiphytes, given their direct exposure to the atmosphere (Hietz et al. 1999; Zotz et al. 2010). For instance, higher nitrogen causes the reduction of chlorophyll content and chlorophyll fluorescence, as well as the reduction of leaf and pseudobulb production for *Laelia speciosa*, for which increased tissue nitrogen content is concurrent with nitrogen isotopic fractionation (Díaz-Álvarez et al. 2015). A plant's ability to accumulate foliar mass is limited by large quantities of nitrogen, because the availability of other nutrients is limited. For instance, excessive availability of nitrogen results in an imbalance of the nitrogen to magnesium ratio in the leaf because the cell releases protons (H^+), which lowers the pH and inhibits chlorophyll production causing the loss of Mg^{2+} (Britto and Kronzucker 2002; Díaz-Álvarez et al. 2015; Mangosá and Berger 1997; Nakaji et al. 2001; Sánchez et al. 2000; Wortman et al. 2012). It is thus important to characterize the nitrogen deposition in the natural habitat of this species.

Environmental changes, such as variation in air temperature and available water, have been studied over multiple years by determining the natural abundance of some stable isotopes in the vegetation. For instance, H, C, and O isotopes of cactus thorns and tree rings indicate the occurrence of droughts in the Sonoran Desert and temperature and precipitation variations in Europe (Cufar et al. 2014; English et al. 2007, 2010; West et al. 2006). Also, changes in nitrogen deposition have been determined from $\delta^{15}N$ of tree rings in the tropics (Hietz et al. 2011; Van Der Sleen et al. 2015). In this respect, some orchids, including *L. speciosa*, produce one pseudobulb per year, which remains attached to the plant for several years (Soto-Arenas and Solano-Gómez 2007). Thus, these storage organs of water, C, and other nutrients could be useful to reconstruct the interannual variation of nitrogen deposition over the plant's lifespan (Dressler 1981; Ng and Hew 2000; Soto-Arenas and Solano-Gómez 2007).

Leaf, pseudobulb, and root C and N content, C:N ratio, as well as the natural abundance of ^{15}N were determined for individuals of *L. speciosa* growing in a city and growing in an oak forest in order to compare the signal that nitrogen deposition had on the tissue composition of this orchid at

each site. In addition, series of annually produced pseudobulbs were analyzed to determine whether this orchid can record the historical changes on nitrogen deposition at either site.

Materials and methods

Study sites

The effect that atmospheric deposition had on tissue nitrogen composition was evaluated for the orchid *Laelia speciosa* from two contrasting sites in central Mexico. The first site was the Instituto de Investigaciones en Ecosistemas y Sustentabilidad, Universidad Nacional Autónoma de México (19°38′55.9″N; 101°13′ 5″W), located in the city of Morelia whose population is 800,000 inhabitants. Here, the mean annual temperature and annual precipitation are 18.3 °C and 773 mm, respectively, and dominant winds blow from the southwest and northwest (Instituto Nacional de Estadística y Geografía 2013; Servicio Meteorológico Nacional 2012). Orchids that had been rescued from a road construction site in 2004 were kept on the original oak branches of *Quercus deserticola* Trel. (Fagaceae) from which they were collected and placed inside a shadehouse. This shadehouse consists of a metallic structure surrounded with a shade-cloth (85 % transmittance), including the top and sides, which allows the free movement of water and gases, while simulating a canopy leading to a temperature difference of up to 4 °C below ambient at midday.

The second site was one of the last natural refuges of *Laelia speciosa* in Mexico, an oak forest that is located at the Cerro el Olvido, Tzintzuntzán, Michoacán (19°37′59″N, 101°29′09″W, 2361 m; García-Cruz et al. 2003). The dominant species at this site is *Quercus deserticola* which is the most common phorophyte for *L. speciosa* (Soto-Arenas 1994). Oak trees in this forest reach 10 m in height and grow in rocky, clay or clay-loam soils (unpublished observations). The mean annual temperature at Cerro el Olvido is 16.1 °C and the annual precipitation is 758 mm, with a rainy season from May to September, and the dominant winds blow from the southwest and the northwest (Servicio Meteorológico Nacional 2012).

Characterization of the atmospheric nitrogen deposition

Given that no infrastructure exists for measuring atmospheric pollution in the study region, the prevalent nitrogen deposition at each site was assessed by means of mosses. Indeed, mosses have been broadly utilized as bioindicators of atmospheric nitrogen deposition because their nitrogen content is correlated with the rates of deposition and

because their isotopic fractionation during assimilation is null, reflecting the $\delta^{15}\text{N}$ values of the prevalent atmospheric deposition (Bragazza et al. 2005; Power and Collins 2010; Wilson et al. 2009; Zechmeister et al. 2008). In particular, the genus *Braunia*, which is widely distributed in various Mexican ecosystems including urban environments, has shown to be an adequate bioindicator of atmospheric nitrogen deposition (Arciga-Pedraza 2010; Cárdenas and Delgadillo 2009). In order to determine the $\delta^{15}\text{N}$ values of the atmospheric nitrogen deposition as well as to determine the differences in rates of deposition by means of the nitrogen content of the mosses, samples of the moss *Braunia* sp. growing on the same oak branches that the orchids and less than 15 cm away of them in the oak forest and in the city were collected. In addition, to preclude any contamination from organic material from the phorophyte, i.e., the so called canopy soil, moss samples were also collected from exposed granite rock in the oak forest or exposed concrete in the city. The moss samples were analyzed as described below.

Plant material

Laelia speciosa (Kunth) Schltr is a sympodial epiphytic orchid with big and showy flowers, pink to lilac-purple petals, and a white lip. Flowers are produced during the spring. This orchid grows in sub-humid temperate climates, between 1250 and 2500 m (Soto-Arenas and Solano-Gómez 2007). The most recent leaf, pseudobulb, and root were collected from 4 individuals at each site, whose leaf and root length ranged from 8 to 12 and 5 to 10 cm respectively. In the oak forest, orchids were collected from the outer portion of trees where canopy soil cannot be formed given a lack of plant cover. Indeed, these plants were not in contact with decomposing plant material and were at a minimum height of 5 m above the ground and a minimum radial length of 3 m from the stem of *Q. deserticola*.

Interannual atmospheric nitrogen deposition

In order to assess whether the nutrient-storing pseudobulbs of *L. speciosa* can record the changes of atmospheric nitrogen deposition over multiple years, series of consecutive pseudobulbs comprised by the ten most recent organs were collected for 4 individuals from each site (Fig. 1) for conducting the C and N analyses described below.

Carbon and nitrogen content and isotopic $\delta^{15}\text{N}$

Plant material was dried at 80 °C in a gravity convection oven until reaching constant weight (Díaz-Álvarez et al. 2015; Drennan 2009). The dried plant samples were ground to a fine powder in a ball mill (Retsch MM300; Retsch,

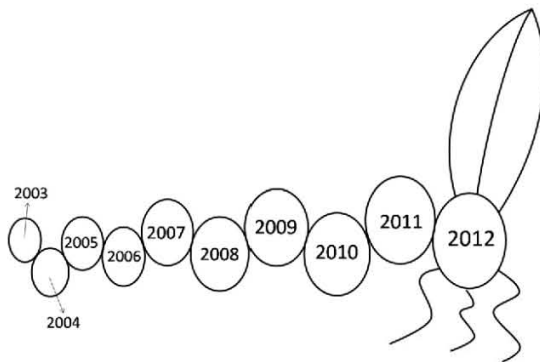


Fig. 1 Morphology of *Laelia speciosa* showing the series of ten consecutive annually produced pseudobulbs. The most recent pseudobulb has been formed during the past year's growing season. The number inside each pseudobulb indicates the year it was formed

Vienna, Austria), wrapped into tin capsules (Costech Analytical, Inc. Valencia, California, USA), and weighed with a microbalance (0.01 mg, Sartorius, Göttingen, Germany). For each sample, the carbon and nitrogen content, as well as their $\delta^{15}\text{N}$ values were determined at the Stable Isotope Facility, University of Wyoming (Laramie, Wyoming, USA), with a Carlo Erba EA 1110 elemental analyzer (Costech Analytical Inc., Valencia, CA, USA) attached to a continuous flow isotope ratio mass spectrometer (Finnigan Delta Plus XP, Thermo Electron Corp, Waltham, MA). Nitrogen isotope ratios, reported in parts per thousand, were calculated relative to atmospheric air standards. The natural abundances of ^{15}N were calculated as:

$$\delta^{15}\text{N}(\text{‰ versus air}) = (R_{\text{sample}}/R_{\text{standard}} - 1) \times 1000$$

where, R_{sample} and R_{standard} is the ratio of $^{15}\text{N}/^{14}\text{N}$ for the sample and standard respectively. (Ehleringer and Osmond 1989; Evans et al. 1996). The analytical precision for $\delta^{15}\text{N}$ was $\pm 0.06 \text{‰}$ (SD).

Statistical analyses

Data normality and homoscedasticity were confirmed before conducting the following statistical analyses. Atmospheric nitrogen deposition, characterized by means

of the moss *Braunia* sp. from the two sites, was analyzed with a one-way ANOVA followed by pairwise Holm-Sidak post hoc tests ($P < 0.05$). The effects of the site of origin (two levels) on the nitrogen content, C:N ratio, and $\delta^{15}\text{N}$ in *L. speciosa* organs (three levels) were analyzed by means of a two-way ANOVA followed by pairwise Holm-Sidak post hoc tests ($P < 0.05$). In turn, differences between the series of 10 consecutive pseudobulbs from two different sites were analyzed by means of a nested ANOVA followed by Holm-Sidak post hoc tests ($P < 0.05$). All analyses were conducted on R (version 2.12.2, R Core Team R Foundation for Statistical Computing, Vienna, Austria).

Results

Characterization of the atmospheric nitrogen deposition

The nitrogen content of the mosses averaged $1.7 \pm 0.1 \%$ (dry mass basis) regardless of the site (Tables 1, 2). Their C:N ratio averaged 21.5 ± 0.8 and no significant difference was found between sites. The $\delta^{15}\text{N}$ reached $3.3 \pm 0.1 \text{‰}$ in the city, contrasting with the $-4.6 \pm 0.9 \text{‰}$ measured in the oak forest ($P < 0.05$; Fig. 2).

Carbon and nitrogen content and $\delta^{15}\text{N}$ for *Laelia speciosa*

The carbon content of leaves, pseudobulbs, and roots of *Laelia speciosa* in the city reached $44.4 \pm 0.3 \%$ (dry mass basis), it was 8.5 % lower than for orchids in the oak forest ($P < 0.05$; Tables 1, 2). In the city, the carbon content of pseudobulbs was $43.4 \pm 0.2 \%$ and it was 10 % lower than the roots ($P < 0.05$). No significant differences were found between leaves and pseudobulbs ($P = 0.12$) nor between leaves and roots ($P = 0.31$). In the oak forest, the carbon content was similar among organs averaging $45.4 \pm 0.2 \%$ ($P \geq 0.05$).

The nitrogen content of pseudobulbs and roots in the city averaged $0.9 \pm 0.1 \%$ (dry mass basis), and it was lower than for the leaves that reached $1.4 \pm 0.1 \%$ ($P < 0.05$; Fig. 3). The nitrogen content of leaves, pseudobulbs, and roots was different among each other in the oak forest ($P < 0.05$). The nitrogen content of leaves in the oak forest

Table 1 Two-way ANOVA for nitrogen content, carbon content, C:N ratio and $\delta^{15}\text{N}$ of *Laelia speciosa* growing in the city and the oak forest

	df	Nitrogen content		Carbon content		C:N ratio		$\delta^{15}\text{N}$	
		F	P	F	P	F	p	F	p
Organ	2	4.07	0.001	5.75	0.01	21.1	0.001	828	0.02
Site	1	20.6	0.06	6.40	0.02	27.0	0.001	4.59	0.001
Organ \times site	2	0.88	0.43	0.10	0.90	12.9	0.001	3.74	0.04

Table 2 Carbon and nitrogen content (dry mass basis) for plant tissue from an oak forest and from a city in Michoacán, Mexico

	Oak forest		City	
	Carbon (% dry mass)	Nitrogen (% dry mass)	Carbon (% dry mass)	Nitrogen (% dry mass)
<i>Braunia</i> sp.				
Inert substrate	39.1 ± 0.24a	1.57 ± 0.03c	37.6 ± 0.32a	1.62 ± 0.09c
Epiphytic	35.4 ± 0.24a	1.66 ± 0.34c	38.6 ± 0.64a	2.00 ± 0.05c
<i>Laelia speciosa</i>				
Leaves	45.6 ± 0.20b	1.3 ± 0.30d	44.5 ± 0.4b	1.3 ± 0.10d
Pseudobulbs	44.6 ± 0.30b	0.4 ± 0.10e	43.4 ± 0.2b	0.9 ± 0.10f
Roots	46.0 ± 0.10b	0.8 ± 0.10f	45.2 ± 0.2b	0.9 ± 0.10f

Data are shown as mean ± 1 SE (n = 4 individuals). For each element, different letters indicate statistically significant differences from pairwise Holm-Sidak tests ($P < 0.05$)

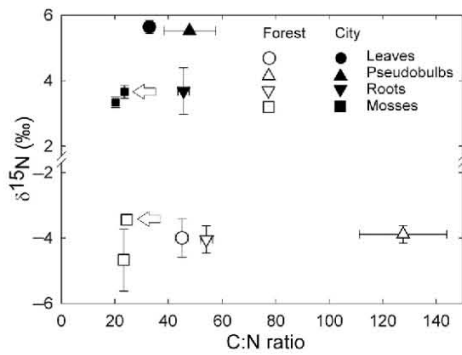


Fig. 2 C:N ratio and $\delta^{15}\text{N}$ for *Laelia speciosa* organs and mosses growing near the orchid on the oak branches and mosses from granite or concrete from two sites in Michoacán, Mexico. Data are shown as mean ± SE (n = 4 individuals per site). The arrows indicate the mosses growing on exposed bare rock at the oak forest or exposed on concrete at the city

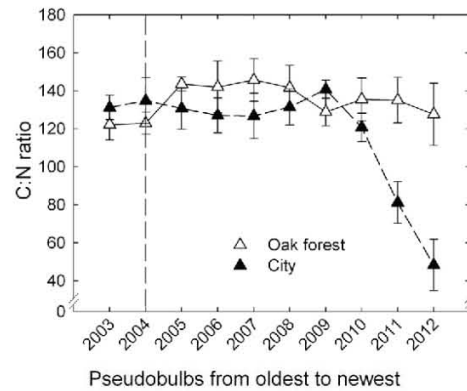


Fig. 4 Carbon to nitrogen ratio for the 10 most recent consecutive pseudobulbs of *L. speciosa* individuals from two sites in Michoacán, Mexico. Data are shown as mean ± SE (n = 4 individuals per site). The vertical dashed line indicates when the rescue plants were transplanted to the city

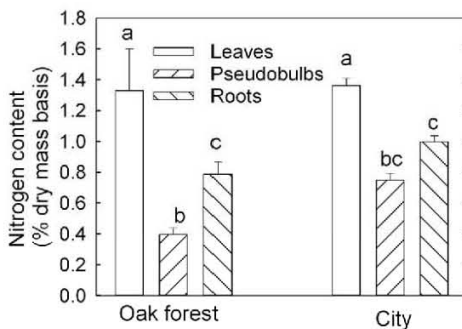


Fig. 3 Nitrogen content (dry mass basis) for the organs of *L. speciosa* from two sites in Michoacán, Mexico. Data are shown as mean ± SE (n = 4 individuals per site). Different letters indicate significant differences ($p < 0.05$)

was similar to the nitrogen content of the leaves in the city and averaged $1.3 \pm 0.2 \%$ (Fig. 3; Table 1). Similar was the case for pseudobulbs and roots whose nitrogen content averaged 0.6 ± 0.1 and $0.9 \pm 0.1 \%$ respectively for both sites (Table 2).

The C:N ratio was similar among the organs of orchids from the city, where it averaged 42.1 ± 1.5 . In turn, the C:N ratio for the organs of orchids from the oak forest was different between pseudobulbs and leaves ($P < 0.01$) and between pseudobulbs and roots ($P = 0.02$). No significant differences were found between leaves and roots, whose C:N ratio averaged 49.7 ± 0.5 ($P \geq 0.05$). The C:N ratio of orchids growing in the oak forest was generally the same as that of orchids growing in the city. Indeed, a C:N ratio of 39.1 ± 2.5 was found for leaves of orchids from both

sites, while it averaged 50.0 ± 2.0 for the roots. An exception was observed for the C:N ratio of 127.6 ± 16.34 for pseudobulbs in the oak forest that was 62 % higher than for the pseudobulbs in the city ($P < 0.001$; Fig. 2; Table 1).

The $\delta^{15}\text{N}$ values were positive for plants from the city and negative for plants from the oak forest ($P < 0.001$). For the plants in the city, differences were found between leaves and roots ($P = 0.01$) and between pseudobulbs and roots ($P = 0.02$). However, no differences were found between leaves and pseudobulbs, averaging 5.6 ± 0.01 ‰. For the orchids from the oak forest, $\delta^{15}\text{N}$ averaged -3.9 ± 0.4 ‰ and no significant differences were found among organs (Table 1; Fig. 2).

Interannual atmospheric nitrogen deposition

The C:N ratios for the entire series of pseudobulbs of the plants from the oak forest, as well as the C:N ratios for the pseudobulbs formed in 2010 and before in the city were similar and averaged 132.4 ± 6.5 (Fig. 4; Table 3). In contrast, for the two most recent pseudobulbs that grew in the city the C:N ratios halved ($P < 0.05$).

The $\delta^{15}\text{N}$ values of pseudobulbs from the oak forest were constant over one decade and averaged -4.4 ± 0.1 ‰ for the entire series ($P \geq 0.05$; Fig. 5). In contrast, the $\delta^{15}\text{N}$ values of pseudobulbs from the city showed a gradual increase from 0.1 ± 1.6 ‰, for the oldest pseudobulb formed at the rescue site in 2003, until a plateau was reached at 4.8 ± 0.3 ‰ for pseudobulbs formed 2007 onwards.

Discussion

Plants tend to increase their photosynthetic rates as nitrogen becomes more available and, in consequence, they accumulate more carbon (Bauer et al. 2004; Brown et al. 1996; Díaz-Álvarez et al. 2015; LeBauer and Treseder 2008; Shangguan et al. 2000). For example, when *L. speciosa* is exposed to simulated nitrogen deposition, its leaf carbon content peaks at 46 % of the dry mass under $20 \text{ kg of N ha}^{-1} \text{ year}^{-1}$, nearly 5 % higher than for plants exposed to $2.5 \text{ kg of N ha}^{-1} \text{ year}^{-1}$ (Díaz-Álvarez et al. 2015). In the present study, the carbon content of the bioindicator *Braunia* sp. and of the orchids, which was the same at both sites, suggest that nitrogen deposition is not high enough to induce significant changes in CO_2 fixation nor changes in the tissue carbon content.

In the present study, the nitrogen content for the moss from both sites was consistent with values measured under low deposition rates, which usually amounts to 1.5 % and up to 2.4 % in some monocots (Chapman 1965; Epstein 1972; 1999; Mills and Jones 1996). The leaves of

Table 3 Nested ANOVA for the elemental and isotopic composition for series of ten consecutive pseudobulbs of *L. speciosa* from the city and the oak forest

	df	C:N ratio		$\delta^{15}\text{N}$	
		F	P	F	P
Year	1	7.30	0.001	4.13	0.045
Year \times site	1	0.69	0.405	99.8	1.49×10^{-5}

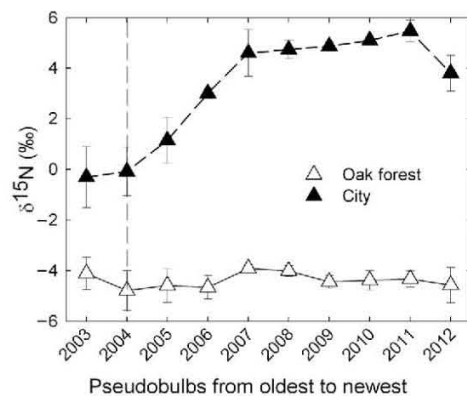


Fig. 5 $\delta^{15}\text{N}$ for the 10 most recent consecutive pseudobulbs of *L. speciosa* individuals from two sites in Michoacán, Mexico (see Fig. 1). Data are shown as mean \pm SE ($n = 4$ individuals per site). The vertical dashed line indicates the time when the rescue plants were transplanted to the city

L. speciosa from both sites also had similar nitrogen content with their values being similar to those of succulent leaves of several species of epiphytic orchids from low nitrogen deposition environments (Cardelús and Mack 2010; Hietz et al. 1999; Mardegan et al. 2011; Wania et al. 2002). The nitrogen content for the plants in this study was substantially lower than for plants from cities with high rates of nitrogen deposition such as London or Mexico City (Arciga-Pedraza 2010; Power and Collins 2010).

While carbon and nitrogen content increase at different rate in response to nitrogen availability, the C:N ratio for the moss and for the leaves and roots of *L. speciosa* was similar at both sites. For the case of the orchid, this parameter was close to the ratio of 56 measured for orchids from the Central Brazilian Amazon, a clean environment (Mardegan et al. 2011). Contrasting with the lack of response from leaves and roots, the C:N ratio of pseudobulbs was different between the sites, which could be the result of a slightly higher accumulation of nitrogen in the city. The lowest nitrogen content of 0.6 % was measured for pseudobulbs in the oak forest because these organs predominantly store

carbohydrates, rather than minerals (Davidson 1960; Hew and Ng 1996; Ng and Hew 2000).

Plants that grow close to sources of pollution reflect the isotopic signals of the pollutants in their tissues (Ammann et al. 1999; Liu et al. 2008; Power and Collins 2010; Redling et al. 2013; Stewart et al. 1995, 2002). For instance, negative isotopic values found in compounds derived from NH_x (agriculture, animal manure, and sometimes vehicle exhaust) result from secondary compounds formed during reactions after emission (Redling et al. 2013). In contrast, compounds derived from the NO_x that are released from industrial activities and fossil fuel burning have positive $\delta^{15}\text{N}$ (Xiao and Liu 2002; Xiao et al. 2012). For example, $\delta^{15}\text{N}$ of -11‰ are found for epiphytic plants growing close to a city in Brazil where the main nitrogenous pollutants are NH_x (-41‰), meanwhile $\delta^{15}\text{N}$ of plants from the field are less negative (-3.1‰ ; Stewart et al. 2002). For epiphytes from a Costa Rican cloud forest (low nitrogen deposition) the $\delta^{15}\text{N}$ reaches -4‰ (Hietz et al. 2002; Wania et al. 2002). In contrast, the $\delta^{15}\text{N}$ for spruce needles from Norway decreases from 2‰ in the proximity of a highway where nitrogen deposition is dominated by NO_x to -3‰ at 1 km into the forest (Ammann et al. 1999). The negative $\delta^{15}\text{N}$ measured in the present study for the mosses and the orchids from the oak forest suggest that these plants assimilated nitrogen from a clean environment (low nitrogen deposition). Indeed, the total emissions in the municipality where the oak forest is located amounted to a 477 ton NO_x and 73 ton NH₃ in 2008 (Sub-Sistema del Inventario Nacional de Emisiones a la Atmósfera de México 2016). In contrast, positive $\delta^{15}\text{N}$ were observed for both the moss and the orchid growing in the city, where the emissions were substantially higher than in the forest in 2008, reaching 57,081 ton NO_x and 2732 ton NH₃ (Sub-Sistema del Inventario Nacional de Emisiones a la Atmósfera de México 2016).

The fact that the $\delta^{15}\text{N}$ was similar among the different organs indicates that isotopic fractionation during the translocation process was very low or absent in plants exposed to the current rates of atmospheric nitrogen deposition at either the forest or the city. These observations contrast with an isotopic fractionation of 3‰ determined for *L. speciosa* growing under a nitrogen deposition of $80\text{ kg N ha}^{-1}\text{ year}^{-1}$ (Díaz-Álvarez et al. 2015).

Physiological responses to climate changes over the lifespan of a plant have been studied with tree rings and cactus spines (English et al. 2007; Hietz et al. 2011; Van Der Sleen et al. 2015). Here, pseudobulb chronosequences developed over a decade effectively recorded the interannual nitrogen deposition at the two sites considered in this study. The C:N ratio observed for the series of pseudobulbs was similar between sites until 2010, when

a significant decrease became apparent for the plants in the city. Presumably, this is the result of 8 years of exposure to urban nitrogen deposition. However, it was not enough to decrease the C:N ratio of pseudobulbs before 2011, suggesting that the rates of nitrogen deposition in the city were only slightly higher than in the oak forest. The $\delta^{15}\text{N}$ values confirmed that the pseudobulbs formed in 2004 and before grew at the rescue site, whereas the more recent pseudobulbs were formed in the city. It was interesting to observe that the first few pseudobulbs formed in city did not show a typical urban $\delta^{15}\text{N}$, but apparently the stored nitrogen that had been assimilated in the rescue site was translocated from the older pseudobulb developed in the city. Over time, the nitrogen from the rescue site was depleted, leaving the urban deposition as the only source of nitrogen for the ensuing pseudobulbs, as evidenced by the positive $\delta^{15}\text{N}$ values of the six most recent pseudobulbs.

Isotopic analyses for *L. speciosa* enabled the distinction between nitrogen deposition in the city and the oak forest. Also, the C:N ratio of the series of pseudobulbs showed that deposition was already slightly higher in the city than in the oak forest, but not enough to effect physiological damage. In turn, the $\delta^{15}\text{N}$ values of the series of pseudobulbs showed that it is indeed possible to track nitrogen deposition and detect possible changes over various years. In addition to land use and climate changes, the risk for *L. speciosa* will increase as the atmospheric nitrogen deposition increases above $20\text{ kg N ha}^{-1}\text{ year}^{-1}$ (Díaz-Álvarez et al. 2015). This is already happening in some regions of Mexico City, where nitrogen deposition rates reach $48\text{ kg N ha}^{-1}\text{ year}^{-1}$ (Secretaría del Medio Ambiente del Distrito Federal 2012). Granted that these alarmingly high rates of nitrogen deposition do not overlap with the distribution range of *L. speciosa*, but deposition rates will most likely exceed $25\text{ kg N ha}^{-1}\text{ year}^{-1}$ by mid-century in the regions of central Mexico where *Laelia speciosa* is endemic (Galloway et al. 2004, 2008; Phoenix et al. 2006). Thus, *L. speciosa* could disappear in the following decades if necessary actions are not taken to reduce the increasing rates of nitrogen deposition in the regions where this epiphytic orchid still grows.

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CAPÍTULO IV

Artículo:

Responses to simulated nitrogen deposition by the neotropical
Epiphytic orchid *Laelia speciosa*

Responses to simulated nitrogen deposition by the neotropical epiphytic orchid *Laelia speciosa*

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ABSTRACT

Potential ecophysiological responses to nitrogen deposition, which is considered to be one of the leading causes for global biodiversity loss, were studied for the endangered endemic Mexican epiphytic orchid, *Laelia speciosa*, via a shadehouse dose-response experiment (doses were 2.5, 5, 10, 20, 40, and 80 kg N ha⁻¹ yr⁻¹) in order to assess the potential risk facing this orchid given impending scenarios of nitrogen deposition. Lower doses of nitrogen of up to 20 kg N ha yr⁻¹, the dose that led to optimal plant performance, acted as fertilizer. For instance, the production of leaves and pseudobulbs were respectively 35% and 36% greater for plants receiving 20 kg N ha yr⁻¹ than under any other dose. Also, the chlorophyll content and quantum yield peaked at 0.66 ± 0.03 g m⁻² and 0.85 ± 0.01, respectively, for plants growing under the optimum dose. In contrast, toxic effects were observed at the higher doses of 40 and 80 kg N ha yr⁻¹. The δ¹³C for leaves averaged -14.7 ± 0.2‰ regardless of the nitrogen dose. In turn, δ¹⁵N decreased as the nitrogen dose increased from 0.9 ± 0.1‰ under 2.5 kg N ha⁻¹yr⁻¹ to -3.1 ± 0.2‰ under 80 kg N ha⁻¹yr⁻¹, indicating that orchids preferentially assimilate NH₄⁺ rather than NO₃⁻ of the solution under higher doses of nitrogen. *Laelia speciosa* showed a clear response to inputs of nitrogen, thus, increasing rates of atmospheric nitrogen deposition can pose an important threat for this species.

Subjects Ecology, Environmental Sciences, Plant Science, Soil Science, Coupled Natural and Human Systems

Keywords Acid rain, Biodiversity loss, CAM, Conservation physiology, δ¹⁵N, Nitrogen pollution, Stable isotopes, Global change, Plant nutrition

INTRODUCTION

Anthropogenic atmospheric nitrogen deposition is considered among the leading global causes of biodiversity loss (Vitousek, 1994; Chapin et al., 2000; Sala et al., 2000). While nitrogen is an essential nutrient for all living organisms, its accelerated release to the atmosphere and ultimate deposition has caused saturation of various ecosystems around the world, leading to significant biodiversity loss by direct toxicity, acidification, and nutrient imbalances between nitrogen and other major nutrients (Aber et al., 1989; Bauer

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et al., 2004; Le Bauer & Treseder, 2008; Bobbink et al., 2010; Templer, Pinder & Goodale, 2012). Most studies regarding the effects of nitrogen deposition on biodiversity have been conducted in the USA and Europe, while studies from megadiverse countries are scant (Bobbink et al., 2010). Considering that the latter countries tend to have developing economies and accelerated industrialization processes, it is urgent to determine the effects that current and future nitrogen deposition rates may have on their local biodiversities (Austin et al., 2013).

A life-form particularly susceptible to the noxious effects of nitrogen deposition are epiphytic plants, such as certain species of orchids and bromeliads, given their reliance on atmospheric sources for nutrients and water (Zotz & Asshoff, 2010; Zotz et al., 2010; Mondragón, Valverde & Hernández-Apolinar, 2015). In this respect, *Laelia speciosa* (Kunth.) Shltr. (Orchidaceae) is an endemic, endangered orchid from central Mexico that has a cultural importance in Michoacán. Not only the plant is collected for its attractive flowers, but juice is extracted from its pseudobulbs and mixed with maize cane pith to produce a paste that is used for the production of sacred art in West Central Mexico (Soto-Arenas & Solano-Gómez, 2007). In addition to extractive pressure, this species faces environmental challenges considering that oak forests, to which this species is restricted, are likely to be severely reduced during the present century (Villers-Ruiz & Trejo-Vazquez, 2000; Rehfeldt et al., 2012). This study assessed whether nitrogen deposition can also pose a threat to this species. However, because current rates of nitrogen deposition are rather low within the area of distribution for *L. speciosa* (Díaz-Álvarez et al., 2014), it was deemed necessary to conduct a shadehouse dose-response experiment to determine the effects of potential future nitrogen deposition on this plant.

Indeed, the purpose of this study was to determine some ecophysiological responses of the endangered neotropical epiphytic orchid *Laelia speciosa* by means of a dose-response shadehouse experiment, in which, the organ production, chlorophyll content, chlorophyll fluorescence, carbon and nitrogen content and isotopic signatures were evaluated for assessing the potential risk that increasing rates of nitrogen deposition pose for this species.

MATERIALS AND METHODS

Plant material

Laelia speciosa is a sympodial epiphytic orchid with big and showy flowers that have pink to lilac-purple petals and a white lip. Flowers are produced during the spring, while an annually produced carbon-storing pseudobulb develops during the summer. *Laelia speciosa* grows in sub-humid temperate climates of central Mexico, between 1250 and 2500 m where its predominant phorophyte *Quercus deserticola* is also found (Soto-Arenas & Solano-Gómez, 2007).

Two-year old plants of *Laelia speciosa* obtained by *in vitro* propagation were transferred into 2L plastic pots containing tezontle (particles were 2–5 cm in diameter), a very porous volcanic rock that is extensively utilized for gardening and hydroponic horticulture given its suitable physicochemical properties (Vargas-Tapia et al., 2008; Yañez-Ocampo et al., 2009). Organic matter was removed from this tezontle by submersion in a SO₄H₂

aqueous solution (50% v/v) followed by a double rinse with deionized, distilled water. The procedure was repeated thrice. The pots were placed in a shadehouse for 2 years at Universidad Nacional Autónoma de México, Campus Morelia (19°38'55.9"N; 101°13'45"W; 1967 m, mean annual temperature of 18.3 °C, annual precipitation 773 mm; Servicio Meteorológico Nacional, 2011), where they were watered every other week until the start of the experiment. A total of 120 plants were selected at random and assigned to one of six groups, each with 20 plants, which received different doses of nitrogen. At the start of the experiment, the plants had 4 pseudobulbs and one leaf (15 cm in length).

Nitrogen deposition scenarios

Starting on 1 October 2011, the plants were watered weekly over two months with 50 ml of a modified Hoagland No. 2 solution from which the nitrogen was omitted to be able to simultaneously supply suitable amounts of nutrients and manipulate the dose of nitrogen dispensed to plants (Hoagland & Arnon, 1950; Nobel & de la Barrera, 2002). At the end of this period, six simulated atmospheric deposition scenarios were applied by adding 1, 4, 8, 16 or 32 mM of NH_4NO_3 to the watering solution, equivalent to 2.5, 10, 20, 40, or 80 kg of $\text{N ha}^{-1} \text{ yr}^{-1}$ respectively. In this respect, a threshold for plant physiological damage has been observed at 20 kg $\text{N ha}^{-1} \text{ yr}^{-1}$, while rates of 40 kg $\text{N ha}^{-1} \text{ yr}^{-1}$ are common in certain parts of Mexico City (Britto & Kronzucker, 2002; Secretaría del Medio Ambiente del Gobierno del Distrito Federal, 2012). The range of doses considered was chosen to establish the threshold for physiological damage for *L. speciosa*, and to determine the effects of deposition rates that are likely to occur during the present century. All amounts were calculated according to the area of the pot of 201 cm^2 . Weekly applications of 50 ml of the experimental solutions were conducted over 26 weeks, from December 2011 to June 2012. This period corresponds to the growth season and reproductive development of *Laelia speciosa* (Halbinger & Soto, 1997; Soto-Arenas, 1994; Soto-Arenas & Solano-Gómez, 2007). Irrigation was carried out on the whole surface of the pot, the coarse substrate allowed the complete drainage of the nutrient solution, simulating what occurs in the canopy.

Physiological responses

Organ production

The emergence of flowers, which are displayed for a few weeks, was recorded weekly. In turn, the total production of new leaves and pseudobulbs, which are persistent, was recorded at the end of the experiment.

Chlorophyll content

Leaf discs were obtained with a cork borer (12-mm in diameter) from 5 plants per nitrogen deposition scenario to determine the concentration of chlorophyll a, chlorophyll b, and total chlorophyll in the plant tissue. The photosynthetic pigments were extracted by macerating leaf tissue with a chilled (3 °C) mortar and pestle in an aqueous solution of acetone (80% v/v) and brought to a final volume of 20 ml. Absorbance was measured at 663 and 646 nm with an EZ 301 spectrophotometer (Perkin Elmer, Waltham, Massachusetts, USA). Chlorophyll concentration was calculated following Lichtenthaler (1987).

Chlorophyll fluorescence (Fv/Fm)

The maximum yield of the photosystem II (the ratio of variable to maximum fluorescence; Fv/Fm) was measured with an Opti-Science 05-30p Fluorometer (Hudson, New Hampshire, USA). Measurements were carried out before dawn, a common practice in plant ecophysiology (Maxwell & Johnson, 2000), for the leaves of five individuals per dose of nitrogen on 29 June and 2 July 2012.

Carbon and nitrogen content and isotopic composition

The leaves of plants growing under different nitrogen doses were collected on 4 July 2012 and dried at 80 °C in a gravity convection oven until reaching constant weight. This temperature has been found to be adequate for tropical succulents, whose membrane proteins can withstand temperatures that are substantially higher than for non-succulent species without incurring in damage (Nobel & de la Barrera, 2002; Drennan, 2009). The dried leaves were ground to a fine powder in a ball mill (Retsch MM300; Retsch, Vienna, Austria), wrapped into tin capsules (Costech Analytical, Inc., Valencia, California, USA), and weighed with a microbalance (0.01 mg, Sartorius, Göttingen, Germany). For each sample, the carbon and nitrogen content, as well as their isotopic proportions, were determined at the Stable Isotope Facility, University of Wyoming (Laramie, Wyoming, USA), with a Carlo Erba EA 1110 elemental analyzer (Costech Analytical Inc., Valencia, California, USA) attached to a continuous flow isotope ratio mass spectrometer (Finnigan Delta Plus XP, Thermo Electron Corp, Waltham, Massachusetts). Carbon and nitrogen isotope ratios, reported in parts per thousand, were calculated relative to the Vienna Pee Dee Belemnite (V-PDB) or atmospheric air standards, respectively. The analytical precision for $\delta^{13}\text{C}$ was $\pm 0.03\text{‰}$ (SD) and $\pm 0.06\text{‰}$ (SD) for $\delta^{15}\text{N}$. The natural abundances of ^{13}C and ^{15}N were calculated as:

$$\delta^{13}\text{C}_{(\text{‰ versus V-PDB})} = (R_{\text{sample}}/R_{\text{standard}} - 1) \times 1000$$

$$\delta^{15}\text{N}_{(\text{‰ versus at-air})} = (R_{\text{sample}}/R_{\text{standard}} - 1) \times 1000$$

where, R is the ratio of $^{13}\text{C}/^{12}\text{C}$ for carbon and $^{15}\text{N}/^{14}\text{N}$ for nitrogen isotope abundance for a given sample (Ehleringer & Osmond, 1989; Evans et al., 1996).

Statistical analyses

The effect of the simulated nitrogen deposition on organ production for *Laelia speciosa* was evaluated by means of a Kruskal-Wallis non-parametric ANOVA, because normality of data was not satisfied, followed by post-hoc Tukey tests ($P \leq 0.05$). In turn, differences in the response of chlorophyll content, chlorophyll fluorescence, carbon and nitrogen content, and $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, which achieved normality, were evaluated with a one-way ANOVA followed by the Holm-Sidak post-hoc test ($P \leq 0.05$). All analyses were conducted on SigmaPlot 12 (Systat Software Inc., San Jose, California, USA).

Table 1 Statistical analyses. Kruskal-Wallis one-way ANOVA and parametric one-way ANOVA for the responses of *Laelia speciosa* individuals growing in a shadehouse under various rates of simulated nitrogen deposition.

	Response to nitrogen dose		
	d.f.	F	P
Leaves	5	8.47	0.001
Pseudobulbs	5	7.04	0.001
Flowers	5	1.94	0.077
Total chlorophyll	5	15.68	0.001
Chla	5	6.67	0.001
Chlb	5	10.47	0.001
Fv/Fm	5	82.5	0.001
Carbon content	5	6.44	0.001
Nitrogen content	5	177.5	0.001
$\delta^{15}\text{N}$	5	15.68	0.001
$\delta^{13}\text{C}$	5	2.65	0.057

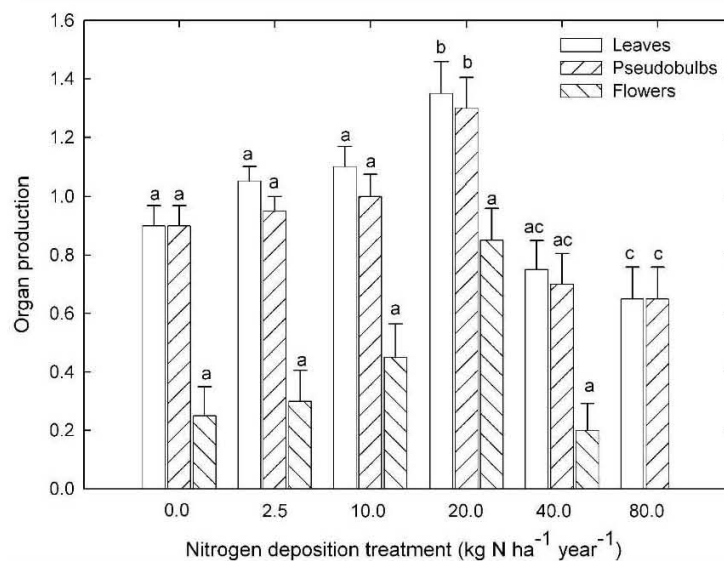


Figure 1 Organ production. Number of new leaves (open bars), pseudobulbs (right hatched bars) and flowers (left hatched bars) that developed on plants of *Laelia speciosa* that were watered with different doses of nitrogen. Data are shown as mean \pm S.E. ($n = 20$ plants per dose of nitrogen). Different letters indicate significant differences ($p < 0.05$) for organs.

RESULTS

Organ production

After 26 weeks of watering the plants with different doses of nitrogen, the production of new organs was greater for those individuals that received 20 kg N ha⁻¹ yr⁻¹ than for those individuals receiving other nitrogen doses (Table 1; Fig. 1). In particular, 1.0 ± 0.1

leaves were produced per plant over the course of the experiment under most doses, except for the plants that received 20 kg N ha⁻¹ yr⁻¹, which produced 35% more leaves ($P \leq 0.001$). Similar was the case for the 0.9 ± 0.1 pseudobulbs produced per plant under most doses, except for the plants that received 20 kg N ha⁻¹ yr⁻¹, which produced 36% more pseudobulbs ($P \leq 0.001$). In contrast, flowering was not significantly influenced by nitrogen dose ($P = 0.077$), with a production of 0.3 ± 0.04 flowers per plant over the course of the experiment (Table 1; Fig. 1).

Chlorophyll fluorescence

The quantum efficiency of photosystem II (Fv/Fm) was similar among the groups of orchids that received up to 20 kg N ha⁻¹ yr⁻¹ amounting to 0.8, while a significant decrease of 23% was observed for plants irrigated with higher concentrations of nitrogen ($P \leq 0.001$; Table 1; Fig. 2A).

Chlorophyll content

Total chlorophyll content for the leaf tissue of *Laelia speciosa* increased as the nitrogen dose increased, peaking at 0.7 ± 0.0 g m⁻² for plants irrigated with 20 kg N ha⁻¹ yr⁻¹ ($P \leq 0.001$; Table 1; Fig. 2B), while the higher doses of nitrogen resulted in a 38% reduction of the pigment. Similarly, the chlorophyll a concentration of 0.5 ± 0.4 g m⁻² was the maximum for plants growing under 20 kg N ha⁻¹ yr⁻¹, and it was 30% lower under all other nitrogen doses ($P \leq 0.001$). In turn, chlorophyll b did not respond to nitrogen, averaging 0.1 ± 0.0 g m⁻² regardless of the dose under which plants grew (Table 1; Fig. 2B).

Carbon and nitrogen content and isotopic composition

The carbon content of *Laelia speciosa* increased with the nitrogen dose peaking at $46.1 \pm 0.3\%$ (dry mass basis) at 20 and 40 kg N ha⁻¹ yr⁻¹ and decreased to $45.2 \pm 0.3\%$ at 80 kg N ha⁻¹ yr⁻¹ ($P \leq 0.001$; Table 1; Fig. 2C).

The nitrogen content for *Laelia speciosa* also increased with the nitrogen dose. For the plants that received up to 10 kg N ha⁻¹ yr⁻¹ the nitrogen content averaged $1.2 \pm 0.0\%$ (dry mass basis), reaching $2.4 \pm 0.0\%$ at 80 kg N ha⁻¹ yr⁻¹ ($P \leq 0.001$; Table 1; Fig. 2D).

The $\delta^{13}\text{C}$ for leaves of *Laelia speciosa* averaged $-14.7 \pm 0.2\text{‰}$ and did not change with the nitrogen dose ($P = 0.057$; Table 1). In contrast, the leaf $\delta^{15}\text{N}$ significantly decreased at higher nitrogen doses. The $\delta^{15}\text{N}$ averaged $0.9 \pm 0.1\text{‰}$ for plants that received up to 10 kg N ha⁻¹ yr⁻¹, a $\delta^{15}\text{N}$ similar to the $\delta^{15}\text{N}$ of $1.1 \pm 0.1\text{‰}$ measured for the NH₄NO₃ utilized for the nutrient solution. The higher doses of nitrogen led to significant decreases of $\delta^{15}\text{N}$, reaching the minimum of $-3.1 \pm 0.2\text{‰}$ for plants growing under 80 kg N ha⁻¹ yr⁻¹ ($P \leq 0.001$; Table 1; Fig. 2E).

DISCUSSION

An intermediate nitrogen dose of 20 kg N ha⁻¹ yr⁻¹ was the most favorable for the production of new organs by *Laelia speciosa*. Lower doses did not improve plant development substantially but higher doses were inhibiting. In this respect, while nitrogen availability may increase leaf production and growth, large quantities of nitrogen limit the

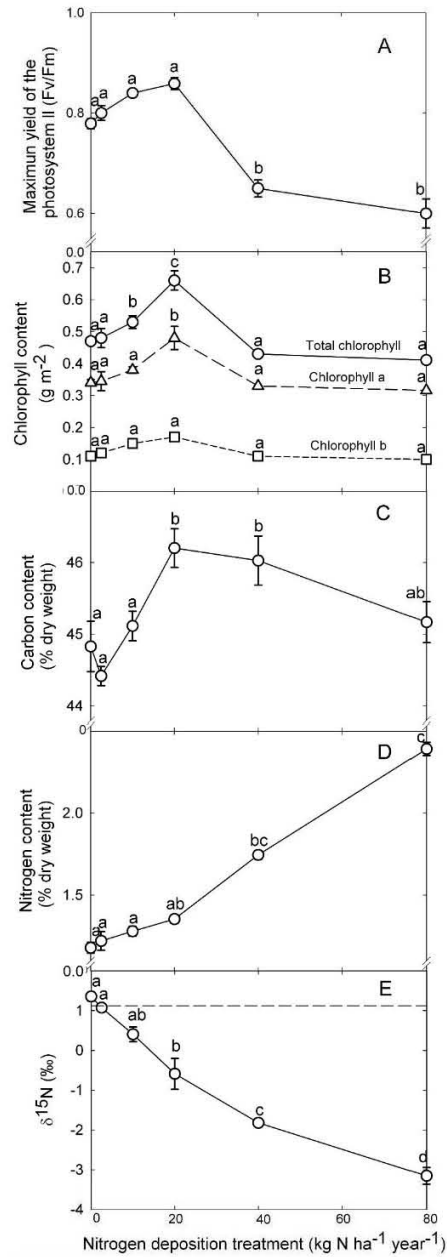


Figure 2 Ecophysiological responses for leaves of *L. speciosa* to simulated nitrogen deposition. (A) Ratio of variable to maximum chlorophyll fluorescence; (B) Tissue content (area basis) for total chlorophyll (circles), chlorophyll-a (triangles), and chlorophyll-b (square); (C) Carbon and (D) nitrogen content (dry mass basis) and (E) $\delta^{15}\text{N}$. Data are shown as mean \pm S.E. ($n = 5$ plants per dose of nitrogen). For each panel, different letters indicate significant differences ($P < 0.05$).

availability of other nutrients, restricting the plant's ability to produce foliar mass (Evans, 1989; Asner, Seastedt & Townsend, 1997; Aber et al., 1998; Sánchez et al., 2000; Zotz & Asshoff, 2010; Díaz-Álvarez et al., 2014). Such behavior was observed for *Laelia speciosa* that showed a substantial reduction in the production of new organs, suggesting noxious effects of the simulated nitrogen deposition. The effect of nitrogen fertilization on *Cymbidium* hybrids is an increased pseudobulb production (Barman et al., 2004). In turn, pseudobulb growth for *Dendrobium nobile* peaks at nitrogen doses of 1.9 mM (Bichsel, Starman & Yin-Tung, 2008).

Total chlorophyll content is proportional to the content of nitrogen in leaves, which typically ranges between 0.4 and 0.5 g m⁻² (Evans, 1989; Nobel, 1999; Nobel & de la Barrera, 2002). Indeed, for *Laelia speciosa*, chlorophyll content increased with the dose of nitrogen, suggesting that this plant was able to assimilate and utilize the supplied nitrogen for the production of photosynthetic pigments. However, the higher doses also resulted in a drastic decrease of the chlorophyll content, as has been documented for other plant species (Baxter, Emes & Lee, 1992; Majerowicz et al., 2000; Lin et al., 2007; Arróniz-Crespo et al., 2008; Ying-Chun et al., 2010). Such a decrease in the chlorophyll content can be explained by the resulting imbalance of the nitrogen to magnesium ratio in the leaf (Nakaji et al., 2001; Wortman et al., 2012). Excessive nitrogen in the cell promotes release of protons (H⁺) and accumulation of phenolic compounds and hydrogen peroxide, as a result, the pH can be altered impeding chlorophyll production and loss of Mg²⁺ (Mangosá & Berger, 1997; Sánchez et al., 2000; Britto & Kronzucker, 2002). Changes in chlorophyll content for *Laelia speciosa* were accompanied by changes in the efficiency of photosystem II, which can be attributed to oxidative stress in the thylakoids that results in the blockage of electron transport to the oxidation site, as a consequence of low available energy for photosynthesis (Maxwell & Johnson, 2000; Poorter, 2000; Hogewoning & Harbinson, 2007; Lichtenthaler et al., 2007; Baker, 2008; Calatayud et al., 2008; Guidi & Degl'Innocenti, 2008; Massacci et al., 2008).

Plants tend to increase their rates of carbon fixation when nitrogen is added (Brown et al., 1996; Bauer et al., 2004; Le Bauer & Treseder, 2008). However, under conditions of chronic nitrogen additions the photosynthetic capacity is inhibited because most of the excess nitrogen is not invested into the primary processes of carboxylation (Brown et al., 1996; Bauer et al., 2004). This also causes an increase and later reduction in carbon content for plants subjected to increasing doses of nitrogen, as was observed here for *Laelia speciosa*. However, the observed $\delta^{13}\text{C}$ values for *Laelia speciosa* which were within the range for CAM plants, did not change under the different nitrogen doses utilized, contrasting with $\delta^{13}\text{C}$ measured for C₃ plants subjected to supplementary nitrogen that became increasingly negative (Raven & Farquhar, 1990; Magalhaes, Huber & Tsai, 1992).

Isotopic discrimination against ¹⁵N increases in plants as the nitrogen availability increases because its assimilation is more energetically costly than for the more abundant ¹⁴N. This so called isotopic effect results in $\delta^{15}\text{N}$ values of the product that are lower than those of the substrate (Evans, 2001; Kolb & Evans, 2003; Ariz et al., 2011). The observed discrimination against ¹⁵N for *Laelia speciosa* leaves has also been observed

for various species, such as *Oryza sativa*, *Pinus sylvestris*, and *Trapa japonica*, species which discriminate between 0.9 and 13‰ when supplied with increasing doses of nitrogen in form of NH_4^+ (Yoneyama et al., 1991; Högberg et al., 1999; Yoneyama et al., 2001; Maniruzzaman & Asaeda, 2012). When the nitrogen source is NH_4^+ , this compound is directly assimilated by the plant cell as amino acids and the involved enzyme, glutamine-synthetase, can discriminate up to 17‰. On the contrary, plants watered with NO_3^- have positive $\delta^{15}\text{N}$ values that have been associated with nitrogen lost via root efflux and exudates or loss of NH_3 through the stomata, processes that favor the lighter isotope (O'Deen, 1989; Yoneyama et al., 2001; Ariz et al., 2011).

Laelia speciosa showed a clear response to increasing doses of nitrogen. Doses of up to 20 kg N ha⁻¹ year⁻¹ enhanced its physiological performance, while higher doses were toxic. The rates of nitrogen deposition in México, where *Laelia speciosa* is endemic, could exceed 25 kg N ha⁻¹ year⁻¹ by mid-century (Galloway et al., 2004; Phoenix et al., 2006; Galloway et al., 2008). As a result, nitrogen deposition poses an actual threat for the persistence of this endangered species as other components of global change represent for many other epiphytic vascular plants (Zotz et al., 2010; Mondragón, Valverde & Hernández-Apolinar, 2015). Future works should consider the effects of nitrogen deposition on wild populations of this and other tropical epiphytic plants. A better understanding of the effects of increasing nitrogen deposition from human activities is of urgent importance, as species ecophysiological response, as those studied here, may be affected, with potentially negative consequences in ecosystem biodiversity and function.

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ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Edison A. Díaz-Álvarez conceived and designed the experiments, performed the experiments, analyzed the data, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.
- Roberto Lindig-Cisneros conceived and designed the experiments, contributed reagents/materials/analysis tools, reviewed drafts of the paper, supervised plant nutrition experiments.
- Erick de la Barrera conceived and designed the experiments, contributed reagents/materials/analysis tools, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.

Supplemental Information

Supplemental information for this article can be found online at <http://dx.doi.org/10.7717/peerj.1021#supplemental-information>.

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CAPÍTULO V

Manuscrito:

Mapping pollution in a megalopolis: the case for atmospheric biomonitors of nitrogen deposition

**Mapping pollution in the megalopolis:
Atmospheric biomonitors of nitrogen deposition**

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Abstract

An increase of nitrogen deposition resulting from human activities is not only a major threat for global biodiversity, but also for human health, especially in highly populated regions. It is thus important and in some instances legally mandated to monitor reactive nitrogen species in the atmosphere. The utilization of widely distributed biological species suitable for biomonitoring may be a good alternative. We assessed the suitability of an ensemble of atmospheric biomonitors of nitrogen deposition by means of an extensive sampling of a lichen, two mosses, and a bromeliad throughout the Valley of Mexico, whose population reaches 30 million, and subsequent measurements of nitrogen metabolism parameters. In all cases we found significant responses of nitrogen content, C:N ratio and the $\delta^{15}\text{N}$ to season and site. In turn, the $\delta^{15}\text{N}$ for the mosses responded linearly to the wet deposition. Also, the nitrogen content ($R^2 = 0.7$), the C:N ratio ($R^2 = 0.6$), and $\delta^{15}\text{N}$ ($R^2 = 0.5$) for the bromeliad had a linear response to NO_x . However, the bromeliad was not found in sites with NO_x concentrations exceeding 212 ppm, apparently of as a consequence of exceeding nitrogen. These biomonitors can be utilized in tandem to determine the status of atmospheric nitrogenous pollution in regions without monitoring networks for avoiding health problems for ecosystems and humans.

Keywords: Biogeochemistry; epiphytic plants; global change; megacities; stable isotopes; urban ecology

Introduction

Nitrogen deposition is one of the most predominant forms of atmospheric pollution (Galloway et al. 2004). This phenomenon results from the release of nitrogenous compounds to the atmosphere, both in cities, and in the country, including oxidized (NO_x) and reduced (NH_x) species, which are highly reactive. Nitrogen deposition causes acidification and eutrophication of aquatic and terrestrial ecosystems, contributes to the proliferation of invasive species leading to changes in ecosystem structure; it is also an important factor for climate change (Sala et al. 2000; Galloway et al. 2004; Phoenix et al. 2006; Duprè et al. 2010; Scherer-Lorenzen et al. 2007; Silva et al. 2015). Nitrogenous pollution is also an issue for human health worldwide. Indeed, in traffic-jammed motorways, high accumulations of NO_x at ground level are harmful by direct inhalation (Reay 2015). NO_x emissions also contribute to the formation of secondary compounds including tropospheric ozone and particulate matter which are responsible in part for 9 million annual deaths worldwide (Landrigan et al. 2018). For the case of Mexico City, a megalopolis with more than 20 million inhabitants, at least 9600 deaths can be attributed annually to atmospheric pollution, resulting from respiratory and heart diseases, and pollutant build up in the nervous system and the brain (Stevens et al. 2008; INEGI 2011). In turn, ammonia emissions from agricultural sources quickly react with other atmospheric pollutants to form airborne particulate matter that can be readily inhaled (Reay 2015). Yet nitrogen is mostly neglected in public policy and the prevalent discourse of global environmental change.

While monitoring nitrogenous pollution is an important issue, the deployment of air quality monitoring networks can be cost prohibitive in regions with developing economies. To fill this gap, an affordable alternative is the use of biomonitors (Diaz-Alvarez et al. 2018).

For instance, throughfall deposition has been determined by the nitrogen content of lichen thalli (Root et al. 2013). Mosses are also widely used as biomonitors that allow covering vast areas, given that their nitrogen content reflects the rates of deposition and their isotopic composition can reflect the possible sources of pollution (Pitcairn et al. 2001; Harmens et al. 2011). A group of special promise for biomonitoring in the neo-tropical region are atmospheric bromeliads, whose succulent tissues allow for yearlong physiological activity, taking up pollution regardless of the seasonal environmental conditions. This is the case for the genus *Tillandsia* (Bromeliaceae), amply distributed in the Americas, which has been utilized for monitoring NO_x pollution given that its nutrition depends exclusively from atmospheric sources (Zambrano et al. 2009; Felix et al. 2016). However, physiological limitations of these atmospheric organisms can confuse the observed deposition, therefore it has been proposed that an assemble of various biomonitors for tracking this type of atmospheric pollution improves biomonitoring (Diaz-Alvarez et al. 2018).

To assess whether the combined use of various biomonitors can provide reliable information on nitrogen deposition we: 1) determined the spatial distribution of nitrogen content and the isotopic composition of the lichen *Anaptychia* sp., the mosses *Grimmia* sp., and *Fabronia* sp., and the bromeliad *Tillandsia recurvata* throughout the Valley of Mexico; and 2) evaluated the suitability as biomonitors of these organisms by comparing their nitrogen with an existing automated atmospheric monitoring network.

Materials and methods

Study area

The Valley of Mexico is located in central Mexico spanning 7500 km², with an average elevation of 2240 m (Calderón and Rzedowski 2001). Annual precipitation ranges

from 600 mm at the center of the valley to 1300 mm in the surrounding mountains. The predominant winds blow from the northeast and northwest (SMN 2016). Mexico City sits at the southern edge of the Valley with a population of 20 million. At the northern edge, Pachuca, the capital of the state of Hidalgo, has a population of 3 million. Additional, settlements interspersed in the valley with industrial or agricultural activities comprise the rest of the 30 million inhabitants of this basin (INEGI 2011).

Nitrogen environment

The Mexico City environmental authority has deployed an air quality network of 16 monitoring stations for wet deposition (Fig. 1). This network collects data during the rainy season from May to November. The nitrogen collected consists of dissolved NO_3^- and NH_4^+ , so that the total nitrogen is the sum of both forms of deposition ($\text{NO}_3^- + \text{NH}_4^+$). This is the dissolved inorganic nitrogen (DIN), biologically available (Liu et al. 2012a). For this study, the rates of wet deposition measured by this monitoring network during 2014 were utilized for the analyses described below. This air quality network also has 27 monitoring stations for measuring the atmospheric NO_x concentration year-round, 24 hours a day. We utilized the cumulative of atmospheric NO_x concentration during the dry season (November 2013 to April 2014) and during the rainy season (from May 2014 to October 2014). Data were obtained from the website of the environmental authority of the Mexico City (<http://www.aire.cdmx.gob.mx/default.php>).

Biomonitoring

We selected four species of three types of atmospheric organisms, the lichen *Anaptychia* sp., the mosses *Grimmia* sp. and *Fabronia* sp., and the bromeliad *Tillandsia recurvata* (L).

These organisms have been utilized as monitors of nitrogen deposition and are abundant in the Valley of Mexico (Zambrano et al. 2009; Harmens et al. 2011; Jovan et al. 2012). We collected tissue samples from 5 individuals of each species growing on different substrates from 36 sites throughout the valley, including urban parks, agricultural sites, and natural protected areas, for the dry season on (8-20 May) and, the wet season 3-15 November of 2014. The distribution of the sites within the basin was determined by the occurrence of the biomonitoring species and by the complex nature of the landscape, which precluded the collection of samples from a regular grid (Wang and Pataki 2010).

The tissue samples were dried at 60 °C in a gravity convection oven until reaching constant weight. The dried tissues were ground to a fine powder in a ball mill (Retsch MM300; Retsch, Vienna, Austria), wrapped into tin capsules (Costech Analytical, Inc. Valencia, California, USA), and weighed with a microbalance (0.01 mg, Sartorius, Göttingen, Germany). For each sample, both the nitrogen content and their isotopic signature were determined at the Stable Isotope Facility, University of Wyoming (Laramie, Wyoming, USA), with a Carlo Erba EA 1110 elemental analyzer (Costech Analytical Inc., Valencia, CA, USA) attached to a continuous flow isotope ratio mass spectrometer (Finnigan Delta Plus XP, Thermo Electron Corp, Waltham, MA). Nitrogen isotope ratios, reported in parts per thousand, were calculated relative to atmospheric air standards. The analytical precision for the $\delta^{15}\text{N}$ was $0.3 \pm 0.07\text{‰}$ (SD). The natural abundances of ^{15}N were calculated as:

$$\delta^{15}\text{N} (\text{‰ versus air}) = (R_{\text{sample}}/R_{\text{standard}} - 1) \times 1000$$

where, R is the ratio of $^{15}\text{N}/^{14}\text{N}$ for nitrogen isotope abundance for a given sample (Ehleringer and Osmond 1989; Evans et al. 2001).

Statistical analyses

We utilized linear regressions to determine the relationship between total wet nitrogen deposition and the nitrogen content (% dry weight), the C:N ratio as well as the $\delta^{15}\text{N}$ of the organisms considered. The same was done for the concentration of atmospheric NO_x. Data accomplished normality in both cases (Skudnik et al. 2015). We performed two way ANOVAs (factors were site and season) followed by a post hoc Holm–Sidak tests ($p \leq 0.05$) to determine differences in the nitrogen content (% dry weight), the C:N ratio, as well as the $\delta^{15}\text{N}$ values of the tissue samples. All statistical analyses were conducted with Sigmaplot 12 (Systat Software Inc. USA).

Geostatistical analyses

We utilized the ordinary Kriging method (a geostatistical gridding tool for irregularly spaced data; Cressie 1988) to determine the rates of wet deposition, and the concentration of NO_x in the sites where no monitoring station was available for the area covered by the monitoring network. Likewise, we modelled the geographical distribution of the nitrogen content, C:N ratio, and the $\delta^{15}\text{N}$ values for the biomonitors, as well as the data from the monitoring network with ArcMap 10 (part of ArcGIS® ESRI, Redlands, USA).

Results

Spatial distribution of nitrogen deposition

The total wet deposition in Mexico City increased from east to west, it ranged from 23 to 45 kg ha⁻¹ year⁻¹ during 2013, and between 24 and 50 kg ha⁻¹ year⁻¹ during 2014 (Figure 2a). We found the highest rates of wet deposition in northwestern of Mexico City. The rest of the valley lacks monitoring stations. The only existing record is from a natural protected

area at the north of the Valley, whose wet deposition is always below $5 \text{ kg ha}^{-1} \text{ year}^{-1}$ (Arciga 2010). For NO_x concentrations, the northern part of Mexico City is the zone with the highest concentrations reaching a cumulative of 291 ppm during the dry season and 189 ppm during the rainy season (Figure 2b). The lowest concentrations of NO_x were found in the south and the east part of Mexico City which reached 60 ppm and 58.6 ppm during dry and rainy season respectively (Figure 2b).

Nitrogen relations of biomonitors

Anaptychia sp.

The nitrogen content of *Anaptychia sp.* ranged from 1.4% (dry mass) in a natural protected area at the north of the Valley to 5.0% in an urban site in Mexico City during the dry season (Figure 3a). We observed the same pattern during the rainy season, in which the nitrogen content ranged from 1.3 to 4.6%. The interaction between site and season was significant for all parameters measured (Table 1). A notable finding is that the highest nitrogen deposition of $50 \text{ kg N ha}^{-1} \text{ year}^{-1}$ did not produce the highest nitrogen content in this lichen, which was observed in a site whose nitrogen deposition reached $31.5 \text{ kg N ha}^{-1} \text{ year}^{-1}$.

The C:N ratio ranged from 7.5 to 39.1, increasing from the urban to the rural sites during the dry season; it ranged from 9.4 to 34.1 during the rainy season. Similarly, the $\delta^{15}\text{N}$ values ranged between -9.4 and 5.2‰ , being more positive in northeastern and downtown Mexico City and in southeastern Pachuca, contrasting with negative values from rural areas (Figure 3b). A linear regression showed no direct relationship between nitrogen deposition and the parameters measured for this lichen ($R^2=0.14$ for nitrogen content, $R^2=0.07$ for the C:N ratio and $R^2=0.16$ for the $\delta^{15}\text{N}$).

Grimmia sp.

We found significant differences between the nitrogen content of mosses from a natural protected area at the north of the valley that reached 1.3% where the deposition was 5.0 Kg N ha⁻¹ year⁻¹, with those of a site at the north of Mexico City of 3.8% where the deposition reached 40 Kg N ha⁻¹ year⁻¹ (Figure 3c). We observed no differences in the nitrogen between for the dry and rainy season (Table 1). A linear regression showed that the atmospheric concentration of NO_x had no effect on the nitrogen content ($R^2 < 0.01$). The C:N ratio was higher in rural areas and decreased significantly in the urban ones, it ranged between 9.5 and 33.6. On the contrary, NO_x concentration had no effect on the C:N ($R^2 < 0.01$).

We found the most negative $\delta^{15}\text{N}$ values of -7.0‰ in a rural area and positive ones of 6.6‰ in urban sites where wet deposition was lower than 35 Kg N ha⁻¹ year⁻¹, exceeding this point $\delta^{15}\text{N}$ became negative reaching -5.3‰ , as occurs with mosses growing at 40 Kg N ha⁻¹ year⁻¹ (Figure 3d; Figure 4). We also observed positive values in the city of Pachuca at the north of the Valley. Season and NO_x concentration showed no effect on the $\delta^{15}\text{N}$ of this moss (Table 1).

Fabronia sp.

The nitrogen content ranged between 1.7 and 3.4% (dry weight) across the Valley. We found no differences between the seasons, but significant differences between the rural areas and some sites in Mexico City (Table 1; Figure 3e). Wet deposition had no effect on the nitrogen content for the mosses from Mexico City. For example, it only increased by 0.3% when nitrogen deposition when from 24.1 to 48.3 Kg N ha⁻¹ year⁻¹. The site and the season were statistical significant (Table 1). The NO_x concentration had no effect on the

nitrogen content of this moss. In addition, wet deposition, nor the NO_x concentration resulted in alterations on C:N ratio, which ranged from 9.0 and 29.5.

The $\delta^{15}\text{N}$ ranged from -6.6 to 7.8‰ . We found the most negative value in a semi-rural site at the north of the Mexico City (Figure 3f), while a $\delta^{15}\text{N}$ of $-6.0 \pm 0.2\text{‰}$ was observed at the south, where the deposition reached $37.6 \text{ Kg N ha}^{-1} \text{ year}^{-1}$. The most positive value was found in the eastern border of Mexico City, where the nitrogen deposition was 33.7 and $27.1 \text{ Kg N ha}^{-1} \text{ year}^{-1}$ during 2013 and 2014 respectively. The urban environment in the city of Pachuca also had a considerable effect on the isotopic composition of this moss, as positive values were the rule. Significant differences were found between rural areas and the urban ones. In contrast, the season showed no effect on the isotopic composition of this moss (Table 1). We found a weak relationship between the rate of nitrogen deposition and the $\delta^{15}\text{N}$ values ($R^2 = 0.2$; Figure 5).

Tillandsia recurvata

The nitrogen content for *Tillandsia recurvata* ranged from 0.8 to 3.6% during the dry season and from 1.0 to 2.2% (dry weight) during wet season (Figure 3g). The highest content was observed in the northern part of Mexico City and was significantly different from the lowest value found in a semirural site in the central part of the Valley. At the same time, bromeliads collected in the city of Pachuca had a similar nitrogen content as those from semirural areas of the Valley (Figure 3g). However, this variation was not due to wet deposition. Instead, it responded positively to the NO_x concentration (Figure 6a). For example, the nitrogen content reached 3.6% in an urban site where the NO_x reached 212.7 ppm, contrasting with the lowest nitrogen content that reached 0.8% in a rural site where NO_x presumably was lower to 60 ppm, based on the lowest recorded concentration in Mexico City. Wet deposition had no effect on the C:N ratio of this bromeliad, but it was strongly

affected by the concentration of NO_x during both seasons (Figure 6b). For example, we found the lowest C:N ratio of 15.9 where the NO_x reached 185.7 ppm, and the highest C:N of 40 where NO_x concentrations presumably were lower than 60 ppm. The δ¹⁵N values for *T. recurvata* were negative in rural areas and became positive in the cities, varying from −5.0 to 4.4‰ during the dry season and −7.7 to 5.1‰ during the wet season (Figure 3h). The δ¹⁵N values responded positively to the NO_x concentration (R²=0.49; Figure 6c), while the wet deposition had no effect on δ¹⁵N values (R²=0.01).

Discussion

The amount of nitrogen deposition observed in the northern part of Mexico City responded directly to the sources of emission. Most remarkable is that the majority of the 70,000 industries registered in 2014 in Mexico City and its metropolitan area are settled in the northern part of the City, which matches perfectly the distribution of both wet and dry nitrogen deposition (SEDEMA 2015). These factories are responsible for the emission of different nitrogenous pollutants including NO_x and NH_x. Another factor that explains the observed distribution of nitrogen deposition is that many important and busy motorways of the Valley are located in this region. Motor vehicles are the most important source of NO_x in Mexico City (SEDEMA 2015; Castro et al. 2017). The predominant deposition in the western part of the city resulted from the wind, which flows in north-to-south direction, transporting part of the nitrogenous emissions to the south of the City. Additionally, nitrogenous emissions from the northern part of the valley including the city of Pachuca and intermediate agricultural fields also contribute with a reactive nitrogen to the total deposition observed in the northern and western parts of the city (SEDEMA 2015).

The nitrogen content of lichens has been utilized as an effective indicator of nitrogen deposition. For example, three species of lichens can record nitrogen inputs below 10 Kg N ha⁻¹ year⁻¹, point at which they saturate (McMurray et al., 2013; Root et al. 2013). We observed a threshold of nitrogen tolerance for lichens from the Valley, when the deposition reached 31.5 kg N ha⁻¹ year⁻¹ above which they could not take up additional nitrogen. We observed a similar pattern for the $\delta^{15}\text{N}$: while the negative values from rural areas were the result of low rates of deposition, in urban areas high rates of deposition resulted in positive isotopic values, however, above the threshold of saturation the isotopic values turned negative, as has been already observed in other species of lichen (Jovan et al. 2012).

The nitrogen content of moss tissues is affected by the rate of deposition and responds to the distance to urban centers (Zechmeister et al. 2008; Liu et al. 2008a,b; Harmens et al. 2011). We also observed an important influence of urban centers for the nitrogen content of mosses from the Valley. Their $\delta^{15}\text{N}$ values are affected by the rates of deposition and the prevalent pollutant (e.g. wet NH_4^+ or NO_3^- ; dry NH_x or NO_x), being more commonly negative in mosses from rural areas (Stewart et al. 2002; Xiao et al. 2010; Liu et al. 2012; Schröder et al. 2014). The observed negative $\delta^{15}\text{N}$ for mosses from rural areas resulted from uptake of NH_4^+ derived from fertilizers and livestock emissions which have characteristic negative $\delta^{15}\text{N}$ values (Liu et al. 2012; Felix et al. 2014a; Skudnik et al. 2015). Mosses from urban areas of the Valley such as Pachuca, as well as some areas of Mexico City where the wet deposition was below 35 Kg N ha⁻¹ year⁻¹ had positive $\delta^{15}\text{N}$, suggesting that they more likely take up NO_3^- derived from NO_x of fossil fuel burning and industrial activities which typically display positive $\delta^{15}\text{N}$ (Felix et al. 2014a).

Mosses take up NH_4^+ preferentially over NO_3^- because less energy is needed in its assimilation (Heldt and Piechulla 2011). Additionally, high deposition rates can cause the inhibition of nitrate reductase, reducing NO_3^- assimilation (Paulissen et al. 2004; Liu et al. 2012a; Liu et al. 2013a). This occurs to some degree when the deposition reaches $10 \text{ Kg N ha}^{-1} \text{ year}^{-1}$, but when it exceeds $30 \text{ Kg N ha}^{-1} \text{ year}^{-1}$ the nitrate reductase become completely inhibited not only precluding for the NO_3^- be uptake leading to its loss by leaching (Gordon et al. 2002; Pitcairn et al. 2006, Liu et al. 2012a,). Nitrate reductase inhibition also has an important effect on the isotopic composition of mosses because it favors the take up of NH_4^+ that typically has negative $\delta^{15}\text{N}$ values (Skudnik et al. 2015). In the Valley of Mexico, mosses $\delta^{15}\text{N}$ became negative when the rates of wet deposition exceeded $35 \text{ Kg N ha}^{-1} \text{ year}^{-1}$, which suggests that nitrate reductase was indeed inhibited, allowing NH_4^+ uptake, a pollutant that represented 35% of the total wet deposition in Mexico City during 2014 (Pitcairn et al. 2006; Wiedermann et al. 2009; SEDEMA 2015). The atmospheric concentration of NO_x had no effect on the nitrogen content nor the isotopic composition of either moss considered here because when NH_3 is the prevalent nitrogenous pollutant in the atmosphere, the nitrogen content of the mosses can increase more than from wet deposition. However, in Mexico City the prevalent nitrogenous gas pollutant is NO_x , suggesting that nitrogen content and the $\delta^{15}\text{N}$ of the mosses responded more to wet deposition than gaseous pollutants (Liu et al. 2012; Redling et al. 2013).

Despite the high rates of wet deposition recorded in Mexico City which exceeded $50 \text{ Kg N ha}^{-1} \text{ year}^{-1}$ in some areas, neither the nitrogen content nor the C:N ratio of *Tillandsia recurvata* were directly affected. This occurs because raindrops cannot be absorbed by the non-absorptive roots of these plants (Schmitt et al. 1989). Instead, the nitrogen was taken up as NO_x by the leaves of this bromeliad because it can absorb particles and gasses from

the air thanks to stomatal gas exchange and the trichomes present in the surface (Schmitt et al. 1989). This is evident from the close relationship found here between the nitrogen content and the NO_x concentration in the Valley. This is similar to what occurs for other atmospheric bromeliads, whose nitrogen content is higher in the vicinity of highways than further away (Figuereido et al. 2004; Abril and Bucher 2008; Santos et al. 2014). The seasonal differences found for the nitrogen content appeared to respond to the phenology of this bromeliad which grows after the rainy season. Indeed, the NO_x were dragged from the atmosphere to the ground surface during rainy season reducing its biological availability (Taiz and Zeiger 2010; SEDEMA 2015).

Both the nitrogen content and the isotopic composition of *T. recurvata* were determined by the predominant anthropogenic activity of the sites where the plant was collected. Bromeliads take up NO_x with positive $\delta^{15}\text{N}$ from industrialized and densely populated areas, contrasting with the negative emissions originated from biogenic sources, the soil and livestock waste (Felix and Elliott 2014; Díaz-Álvarez et al., 2016). The positive $\delta^{15}\text{N}$ found for *T. recurvata* suggests no nitrate reductase inhibition, but it is likely that NO_x concentrations higher than 221 ppm may result in some inhibition of this enzyme. This could be one of the reasons why *T. recurvata* was not found in sites with concentrations of NO_x higher to 221.2 ppm.

The CAM bromeliad *Tillandsia recurvata* remains physiologically active yearlong recording pollution by NO_x during the entire year. But, because it does not record wet deposition, predicting possible rates of deposition only with this bromeliad is difficult. Thus, the combined use of *T. recurvata* with another biomonitor, such as mosses that do record wet deposition, can become a useful tool for determining the status of nitrogenous pollution in regions where air quality monitoring networks are not available, especially

during early stages of deposition when these organisms better respond. This methodology can work on mid-sized cities and surrounding areas where the saturation threshold for these organisms has not been reached. Finally, the utilization in tandem of these organisms can inform an early alert for avoiding health problems for both ecosystems and humans.

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Table 1. Two-way ANOVA for responses of potential biomonitoring organisms growing in the Valley of Mexico.

		d.f.	%N (dry weight)		C:N ratio		$\delta^{15}\text{N}$ (‰)	
			F	P	F	P	F	P
<i>Anaptychia</i> sp.	Site	33	26.16	<0.001	34.78	<0.001	29.04	<0.001
	Season	1	7.83	0.005	10.14	0.002	0.99	<0.001
	Site \times Season	33	3.18	<0.001	3.22	<0.001	2.34	<0.001
<i>Grimmia</i> sp.	Site	31	34.33	<0.001	40.73	<0.001	123.04	<0.001
	Season	1	1.47	0.226	30.02	<0.001	2.95	0.087
	Site \times Season	31	5.27	<0.001	3.25	<0.001	4.95	<0.001
<i>Fabronia</i> sp.	Site	29	24.92	<0.001	15.50	<0.001	46.87	<0.001
	Season	1	2.57	0.11	6.14	0.014	1.44	0.232
	Site \times Season	29	5.80	<0.001	3.93	<0.001	6.62	<0.001
<i>Tillandsia recurvata</i>	Site	21	34.82	<0.001	33.92	<0.001	57.47	<0.001
	Season	1	96.05	<0.001	6.53	0.011	136.29	<0.001
	Site \times Season	21	16.02	<0.001	3.97	<0.001	7.56	<0.001

Figure legends

Figure 1. Localization of the Valley of Mexico. Red and yellow dots represent the spatial distribution of the air quality network stations of the wet deposition and the automatic monitoring network for NO_x, respectively. This network is located mainly in the Mexico City and its metropolitan area. Green triangles represent the sites where biomonitors were collected throughout the Valley. The red line delimits the basin, the white line indicates state division, and the blue line shows Mexico City limits.

Figure 2. Spatial distribution of the total wet deposition in Kg N ha⁻¹ year⁻¹ during 2014 (A), and atmospheric concentration of NO_x in ppm (B). Data is available for public access in the website of the Mexico City government (<http://www.aire.cdmx.gob.mx/default.php>).

Figure 3. Spatial distribution of the nitrogen content (A, C, E, G) and δ¹⁵N values (B, D, F, H) for *Anaptychia* sp. (A, B), *Grimmia* sp. (C, D), *Fabronia* sp. (E, F) and *Tillandsia recurvata* (G, H).

Figure 4. Relationship between wet deposition of ammonium (green circles), nitrate (blue squares), and total deposition, NH₄⁺ + NO₃⁻ (red triangles) during 2014 and the δ¹⁵N values of the moss *Grimmia* sp.

Figure 5. Relationship between wet deposition, of ammonium (green circles), nitrate (blue squares) and total deposition, NH₄⁺ + NO₃⁻ (red triangles) during 2014 and the δ¹⁵N values of the moss *Fabronia* sp.

Figure 6. Relationship between NO_x concentration during the 2014 dry season and the nitrogen content (A), C:N ratio (B), and the δ¹⁵N values (C) of the bromeliad *Tillandsia recurvata*.

Figure 1.

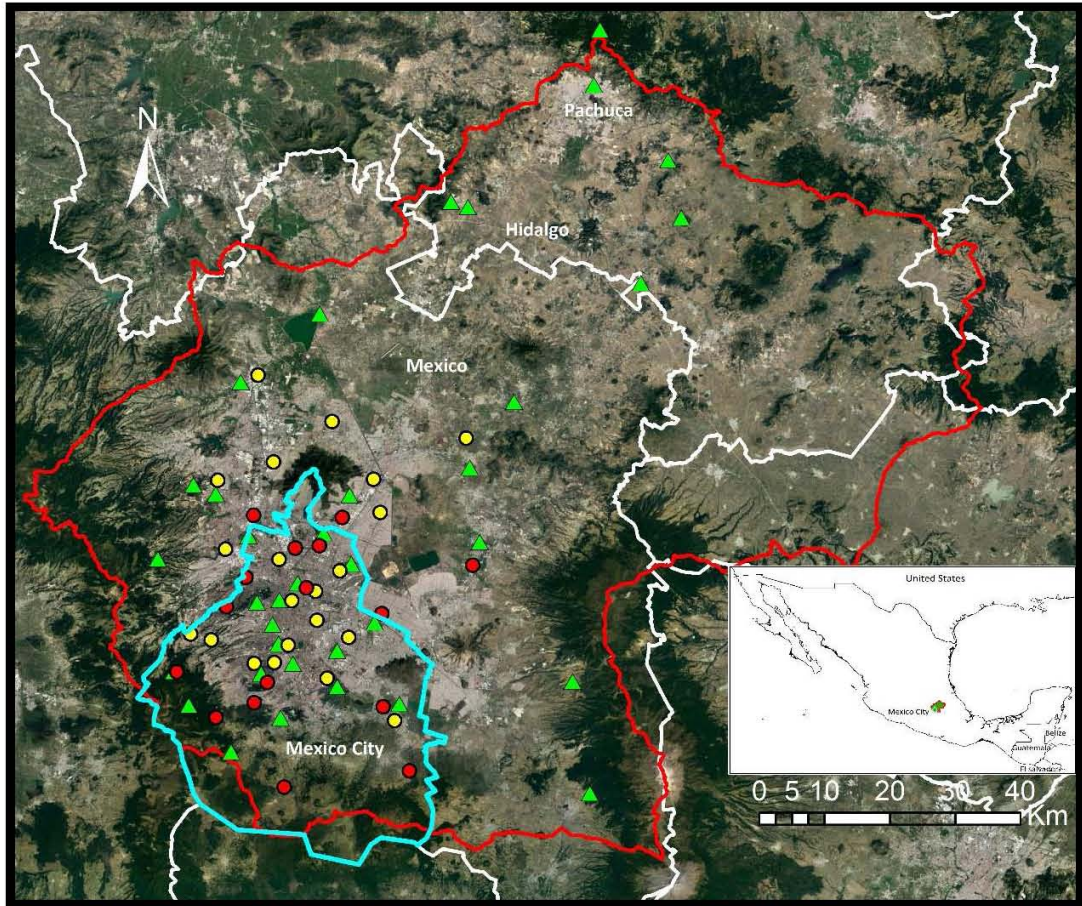


Figure 2

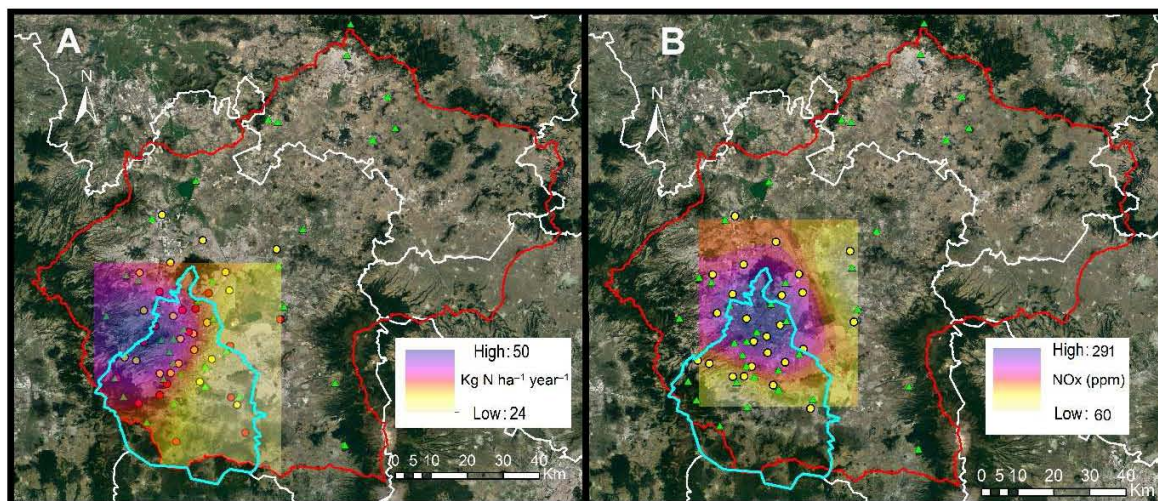


Figure 3.

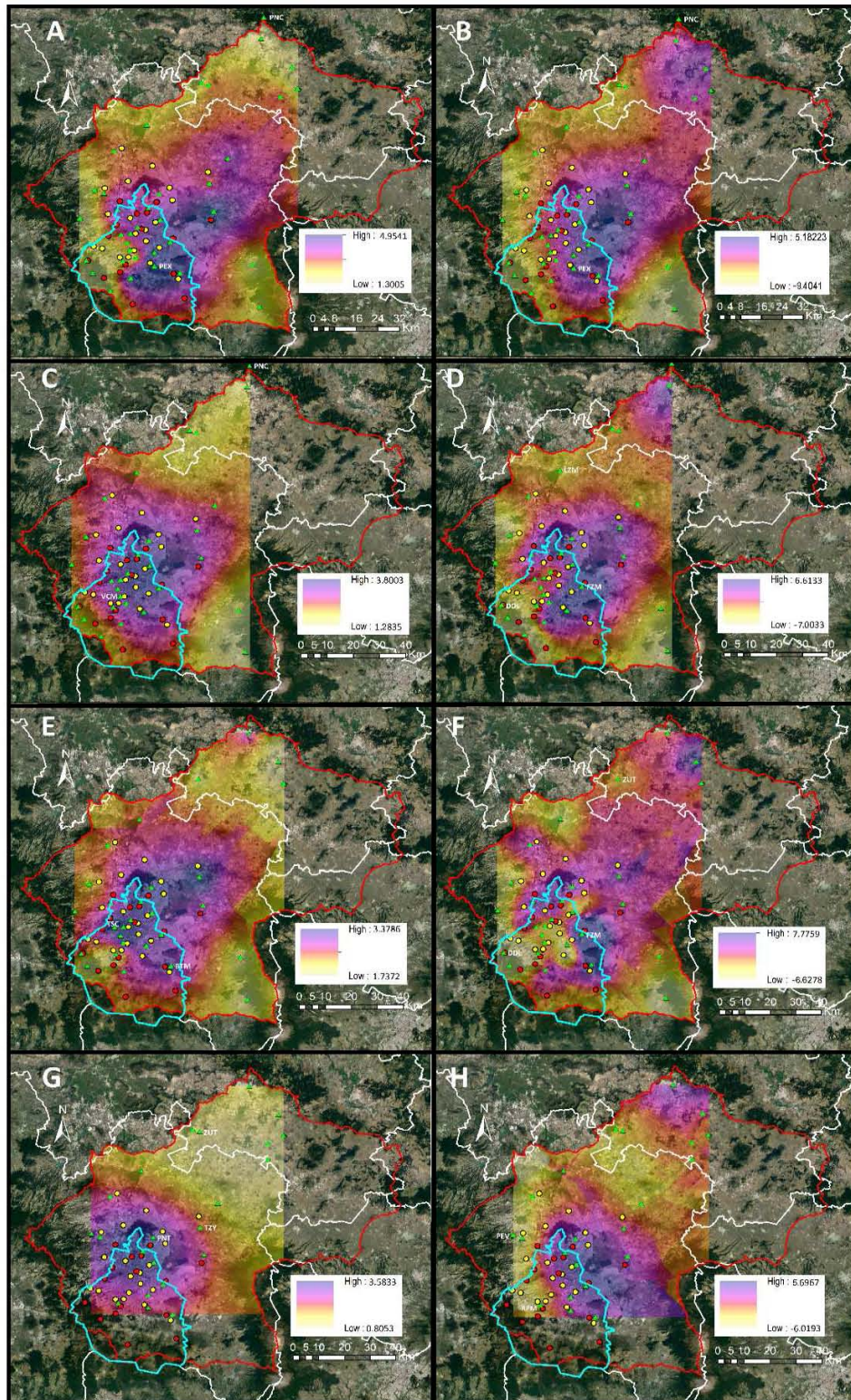


Figure 4.

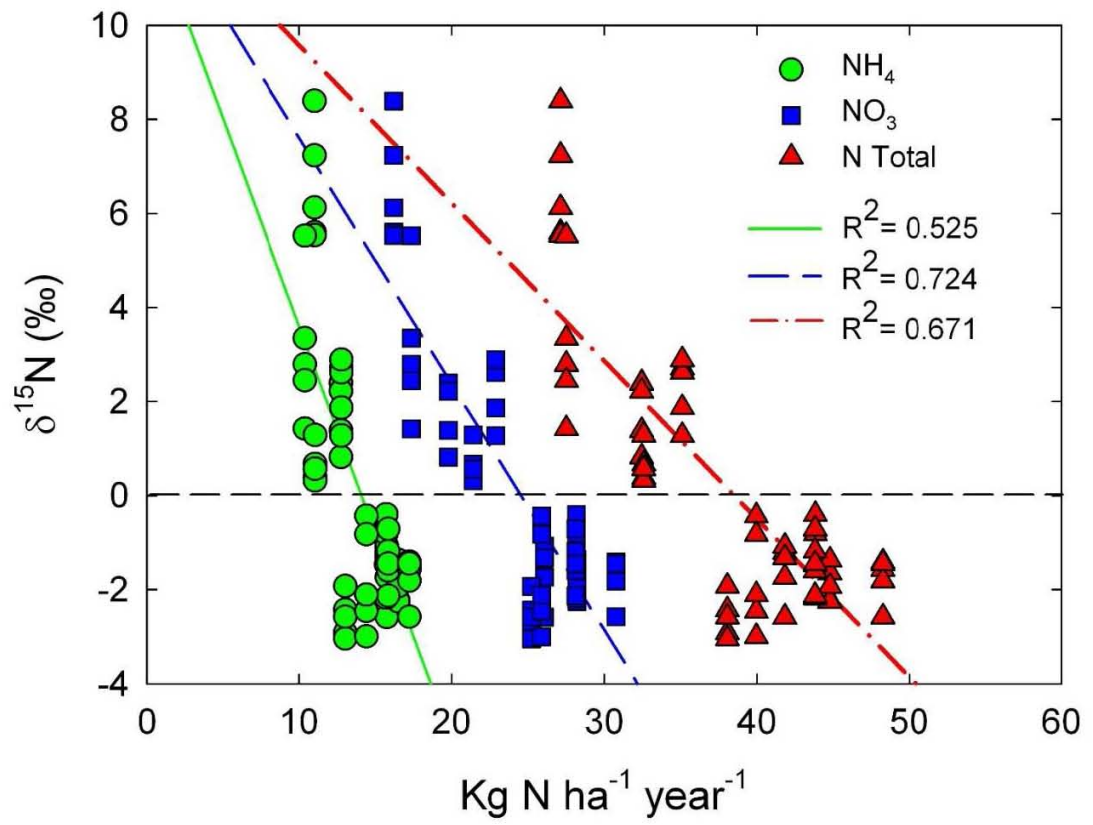


Figure 5.

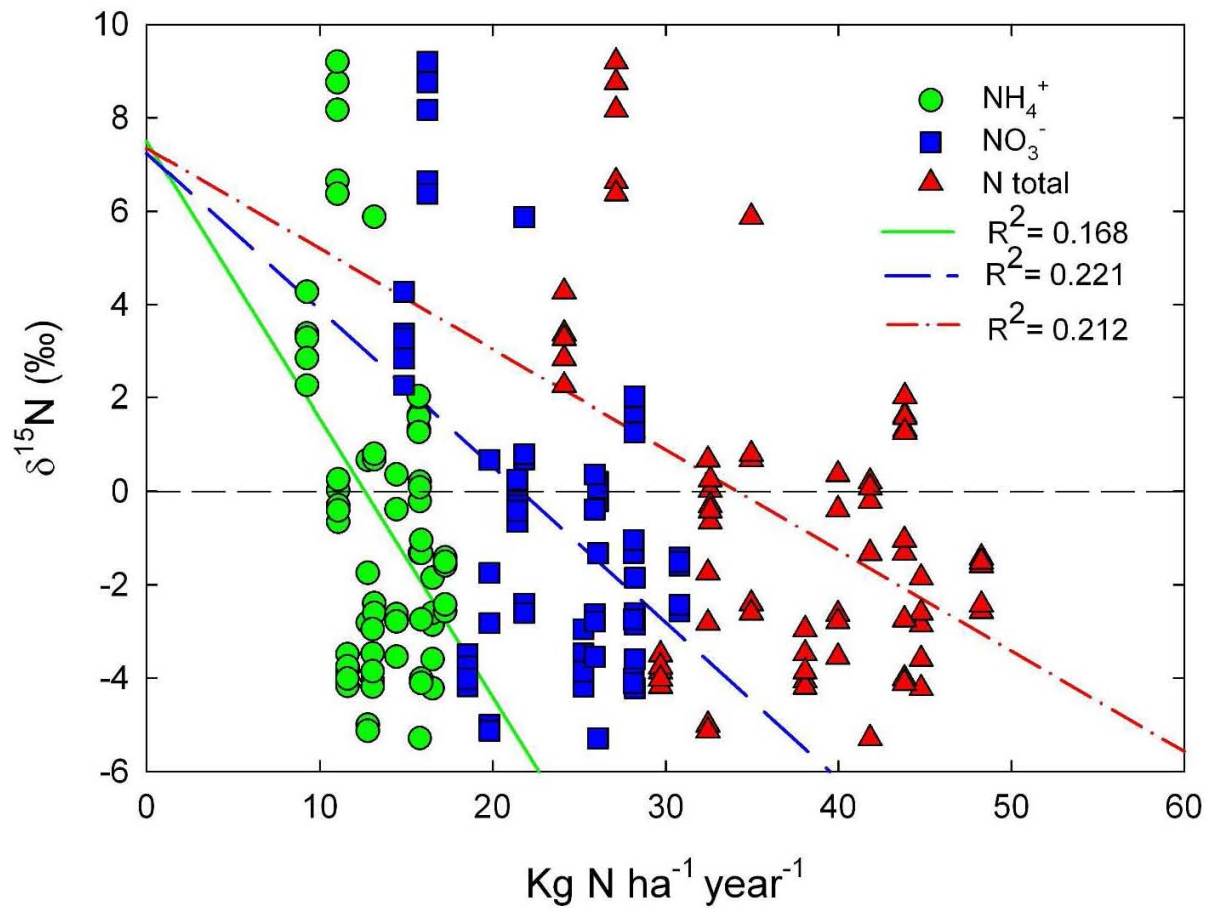
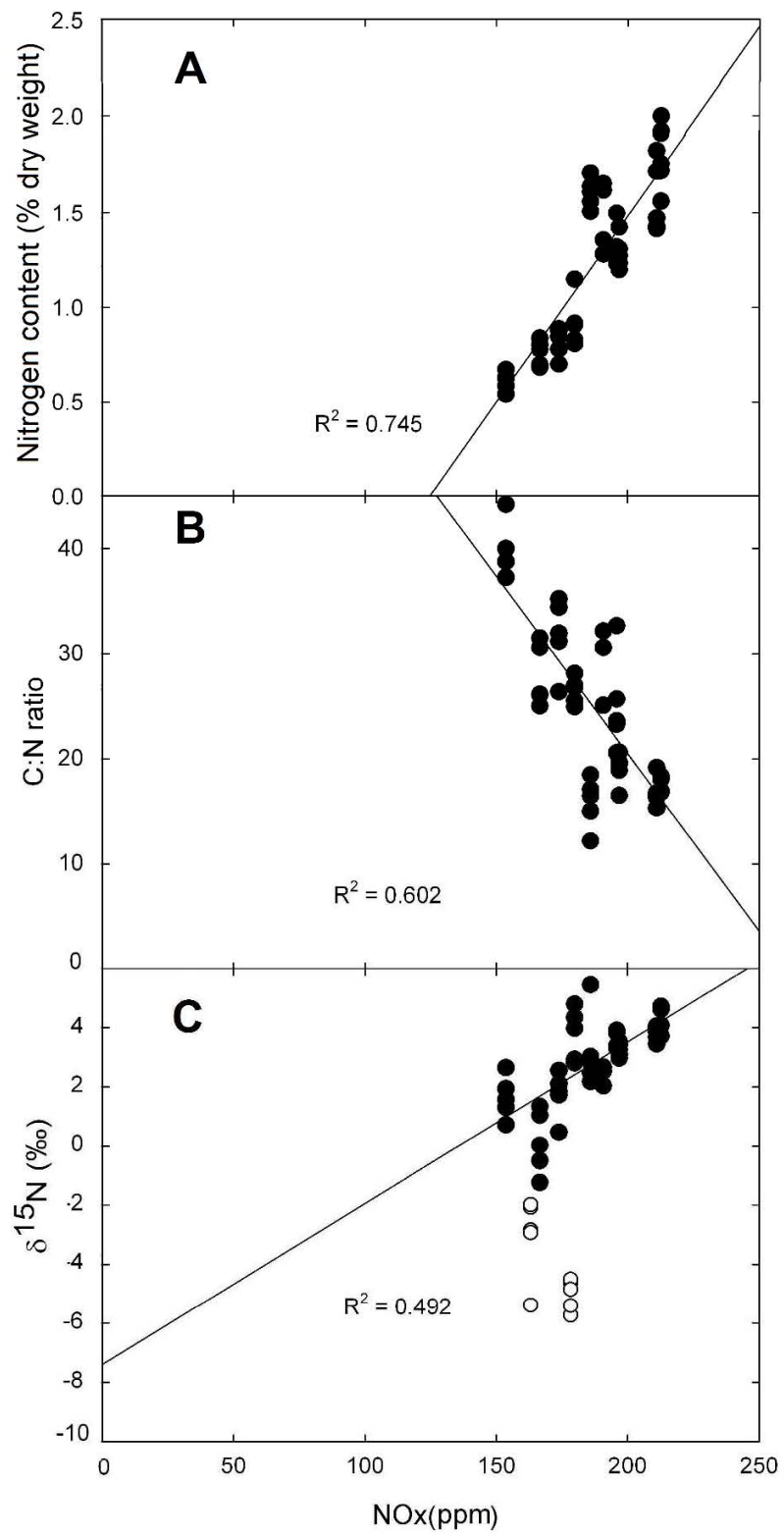


Figure 6.



CAPÍTULO VI

Manuscrito:

Legislación mexicana en materia de contaminación atmosférica,
monitoreo y salud pública

CONTAMINACIÓN ATMOSFÉRICA Y MONITOREO DE LA CALIDAD DEL AIRE EN MÉXICO: NORMATIVIDAD Y CUMPLIMIENTO

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RESUMEN

La contaminación del aire es un problema creciente en economías en desarrollo que afecta directamente la salud y el ambiente. México ha establecido una serie de leyes y normatividades en materia de contaminación atmosférica para proteger la salud de sus habitantes. También, ha establecido una reglamentación clara para el monitoreo de la calidad del aire. Aquí se compila la normatividad existente en materia de contaminación atmosférica. Dentro de la normatividad federal se encuentran 14 normas en materia de emisiones por fuentes fijas y 10 en materia de fuentes móviles. Adicionalmente existen 12 normas en materia de monitoreo ambiental y salud de la población entre las que destaca la NOM-156-SEMARNAT-2012. Esta norma dicta que aquellos centros urbanos que cumplan ciertas características deben tener sistemas de monitoreo de la calidad del aire. Un análisis de su cumplimiento reveló que quince zonas metropolitanas y 72 zonas conurbadas no cumplen con lo normado. También, encontramos que 352 centros urbanos tienen emisiones de contaminantes criterio superiores a las 20 mil toneladas anuales permitidas, de las cuales 228 no cuentan con sistemas de monitoreo de la calidad del aire. Por lo anterior, al menos 20 millones de personas de todo México no cuentan con cubrimiento de sistemas de monitoreo debido a que viven en poblaciones que según la NOM-156 están obligadas a monitorear la calidad del aire y no lo hacen. Se recomienda realizar una revisión de los centros urbanos en incumplimiento para determinar las causas y acciones a tomar, así como considerar ampliar el conjunto de contaminantes monitoreados de importancia en salud pública y ambiental.

***Palabras clave:** Contaminación ambiental; sistemas de monitoreo; cambio climático; salud pública; zonas metropolitanas*

INTRODUCCIÓN

La contaminación atmosférica ha sido un problema de gran importancia desde la revolución industrial. De acuerdo al Programa de las Naciones Unidas para el Medio Ambiente cerca de 7 millones de personas mueren cada año en el mundo debido a la mala calidad del aire y la contaminación atmosférica (OMS, 2014; ONU, 2016). El Banco Mundial considera que la contaminación del aire es el cuarto factor de riesgo para la salud a nivel mundial, además la atención médica y el ausentismo por enfermedades asociadas causa la pérdida de 250 mil millones de dólares cada año (Banco Mundial, 2016). En décadas recientes la atención gubernamental sobre la contaminación ambiental ha tomado creciente importancia en países con economías emergentes. Tal es el caso de México, cuyo aumento poblacional ha venido acompañado del incremento de la industria y del uso de automóviles sobre todo en los crecientes centros urbanos de todo el país, donde las emisiones de contaminantes tienen impacto directo en la salud. De hecho, la contaminación causa cerca de 20,000 muertes cada año, de las cuales al menos 9,000 se registran en la zona metropolitana del Valle de México producto de enfermedades cardíacas y respiratorias (Stevens et al., 2008). De igual forma, se han encontrado partículas de magnetita en el cerebro y en el sistema nervioso de personas que vivieron en la Ciudad de México durante algún periodo del siglo XX (Maher et al., 2016). La magnetita es un contaminante típico derivado de algunos procesos de combustión y puede llegar a ser extremadamente tóxica (Maher et al., 2016). La contaminación atmosférica también tiene efectos negativos en el ambiente, no solo en el sitio de emisión sino también en zonas remotas ya que puede ser transportada grandes distancias por las corrientes de aire y depositarse en la lluvia o por acción de la gravedad, causando pérdida de la biodiversidad y el deterioro de recursos naturales de consumo humano básico como el agua (Persson et al., 2010; Simkin et al., 2010).

El derecho humano a un ambiente sano y equilibrado es considerado en el artículo 4o de la Constitución Política de México, en la Ley General del Equilibrio Ecológico y la Protección del Ambiente, y en la Ley General de Cambio Climático. Por ello, en cumplimiento de estas regulaciones, se generan informes nacionales de la calidad del aire y de emisiones contaminantes a la atmósfera para conocer la calidad del aire que respiran los mexicanos. También, para lograr lo contemplado en estas leyes se crearon las Normas Oficiales Mexicanas (NOM) en las cuales están establecidos los criterios y regulaciones de

cuantificación y medición de la emisión de contaminantes a la atmósfera. Adicionalmente, el Instituto Nacional de Ecología y Cambio Climático en conjunto con la Secretaría de Medio Ambiente y Recursos Naturales inauguraron en 2016 el Sistema Nacional de Información de la Calidad del Aire (INECC, 2016). Este sistema constituye una plataforma de internet en la cual se reúnen los datos de la calidad del aire de gran parte de la república mexicana. Este sistema de acceso público tiene como objetivo informar a la población en general sobre la calidad del aire.

Los objetivos de este estudio son analizar la normatividad en materia de contaminación atmosférica y evaluar el cumplimiento por parte de las autoridades locales, regionales y nacionales de los diferentes campos de aplicación de la NOM-156-SEMARNAT-2012, que es la norma encargada de regular el monitoreo de la calidad del aire en los centros urbanos de todo el país. Así mismo, usando como base los resultados de nuestro análisis y estudios previos en el tema, presentamos propuestas para contribuir con el mejoramiento de la normatividad vigente en materia de la calidad del aire y su aplicación.

METODOLOGÍA

Las normas oficiales mexicanas vigentes se encuentran especificadas en el Catálogo de Normas Oficiales Mexicanas reunidas en el Sistema Integral de Normas y evaluación de la Conformidad (SINEC, disponible en: www.sinec.gob.mx/SINEC/). Para encontrar las normas relevantes para este análisis realizamos una búsqueda utilizando las palabras clave “control de emisiones”, “salud de la población”, “monitoreo” y “calidad del aire” en dicho catálogo. Para determinar el grado de cumplimiento de la NOM-156-SEMARNAT-2012 (NOM-156 en el resto del texto) se consultaron diferentes herramientas incluyendo el Sistema Nacional de Información de la Calidad del Aire (SINAICA, disponible en: <http://sinaica.inecc.gob.mx/>), de donde se obtuvieron los datos en tiempo real de la calidad del aire de las estaciones de monitoreo automáticas activas en México. Del SINAICA también se obtuvo información de las estaciones inactivas o con datos insuficientes. Así mismo, del Informe Nacional de la Calidad del Aire 2014 (SEMARNAT, 2013) se obtuvo la información concerniente a la calidad del aire en México y el número y estado de las redes de monitoreo atmosférico en el territorio nacional (INECC, 2015). La información recabada

de estas fuentes se resume en un cuadro en el que se muestran las zonas metropolitanas y la situación de los sistemas de monitoreo de cada una (Cuadro 1). Posteriormente, se modeló el estado de los sistemas de monitoreo de la calidad del aire de las zonas conurbadas y metropolitanas con ArcMap 10.2 (parte de ArcGIS® Sri, Redlands, EE.UU.). La identidad de dichos centros urbanos (zonas conurbadas y zonas metropolitanas) que son sujetos obligados al cumplimiento de la NOM-156 fue obtenida del Instituto Nacional de Estadística y Geografía y de la Consejo Nacional de Población (CONAPO, 2012; INEGI, 2017).

MARCO NORMATIVO DE LAS EMISIONES Y LA CONTAMINACIÓN ATMOSFÉRICA EN MÉXICO

México cuenta con diferentes instrumentos normativos que regulan la emisión de contaminantes a la atmósfera. En la Constitución Política, el artículo 4o reconoce el derecho humano a la salud y a un medio ambiente sano. También en el artículo 5o de la Ley General del Equilibrio Ecológico y la Protección al Ambiente se establece la prevención de la contaminación del aire. Mientras que en el artículo 2o de la Ley General de Cambio Climático se establece como objetivo garantizar el derecho a un medio ambiente sano. Por su parte la Ley General de Salud en el título séptimo dicta que corresponde a las autoridades de salubridad determinar los valores de concentración máxima permisibles para el ser humano de los contaminantes atmosféricos. Así, se ha establecido la reglamentación de los límites permisibles de emisión y concentración de contaminantes criterio primarios (O₃, CO, SO₂, NO₂, Pb, PST, PM₁₀ y PM_{2.5}). Las regulaciones incluyen las emisiones provenientes de diferentes actividades como procesos industriales y el transporte. Además, incluyen lineamientos para su medición con el fin de garantizar la protección de la población y su derecho fundamental a un ambiente sano. Los elementos normativos que regulan las diferentes actividades que causan contaminación atmosférica están contemplados en las Normas Oficiales Mexicanas (NOM) en las cuales se presentan las regulaciones técnicas que contienen la información, requisitos, especificaciones, procedimientos y metodologías que establecen parámetros de evaluación y medición de las emisiones de contaminantes a la atmósfera, en materia de emisiones provenientes de fuentes móviles se encuentran 10 normas oficiales, en materia de emisiones de fuentes fijas son 14 normas oficiales, mientras que en

materia de monitoreo ambiental y salud de la población son 12 las normas oficiales entre las que se destaca la NOM-156-SEMARNAT-2012. (Cuadro 1).

Cuadro 1. Tipo de contaminante y normas oficiales mexicanas que regulan su emisión y monitoreo. Las descripciones de las normas están disponibles en el Apéndice 1 de emisiones de fuentes móviles, en el Apéndice 2 de emisiones de fuentes fijas y en el Apéndice 3 de normas de monitoreo y salud de la población.

Contaminante	NOMs de emisiones	NOMs de monitoreo
Monóxido de carbono (CO)	NOM-040-ECOL-2002	NOM-021-SSA1-1993
	NOM-041-SEMARNAT-2015	NOM-034-SEMARNAT-1993
	NOM-042-SEMARNAT-2003	NOM-156-SEMARNAT-2012
	NOM-044-SEMARNAT-2006	
	NOM-047-SEMARNAT-1999	
	NOM-048-ECOL-1993	
	NOM-049-ECOL-1993	
	NOM-050-ECOL-1993	
	NOM-076-ECOL-1995	
	NOM-085-SEMARNAT-2011	
NOM-098-ECOL-2002		
Óxidos de nitrógeno (NOx)	NOM-040-ECOL-2002	NOM-023-SSA1-1993
	NOM-041-SEMARNAT-2015	NOM-037-SEMARNAT-1993
	NOM-042-SEMARNAT-2003	NOM-156-SEMARNAT-2012
	NOM-044-SEMARNAT-2006	
	NOM-047-SEMARNAT-1999	
	NOM-050-ECOL-1993	
	NOM-076-ECOL-1995	
	NOM-085-ECOL-1994	
	NOM-086-SEMARNAT-SENER-SCFI-2005	
	NOM-097-ECOL-1995	
NOM-098-ECOL-2002		

	NOM-166-SEMARNAT-2014	
Compuestos azufrados (SO _x)	NOM-039-ECOL-1993	NOM-022-SSA1-2010
	NOM-040-ECOL-2002	NOM-038-SEMARNAT-1993
	NOM-046-ECOL-1993	NOM-156-SEMARNAT-2012
	NOM-051-ECOL-1993	
	NOM-085-ECOL-1994	
	NOM-086-SEMARNAT-SENER-SCFI-2005	
	NOM-098-ECOL-2002	
	NOM-105-ECOL-1996	
	NOM-137-SEMARNAT-2013	
	NOM-148-SEMARNAT-2006	
Plomo (Pb)	NOM-040-ECOL-2002	NOM-026-SSA1-1993
	NOM-098-ECOL-2002	NOM-156-SEMARNAT-2012
	NOM-166-SEMARNAT-2014	
Ozono (O ₃)		NOM-020-SSA1-2014
		NOM-036-ECOL-1993
		NOM-156-SEMARNAT-2012
Partículas suspendidas (PM ₁₀ , PM _{2.5})	NOM-040-ECOL-2002	NOM-025-SSA1-2014
	NOM-098-ECOL-2002	NOM-156-SEMARNAT-2012
Partículas suspendidas totales (PST)	NOM-040-ECOL-2002	NOM-035-SEMARNAT-1993
	NOM-043-ECOL-1993	NOM-156-SEMARNAT-2012
	NOM-085-SEMARNAT-2011	
	NOM-097-ECOL-1995	
	NOM-098-ECOL-2002	
	NOM-105-ECOL-1996	
Hidrocarburos	NOM-040-ECOL-2002	
	NOM-041-SEMARNAT-2015	
	NOM-042-SEMARNAT-2003	
	NOM-044-SEMARNAT-2006	

	NOM-047-SEMARNAT-1999
	NOM-048-ECOL-1993
	NOM-049-ECOL-1993
	NOM-050-ECOL-1993
	NOM-076-ECOL-1995
	NOM-166-SEMARNAT-2014
Neblinas de ácido sulfúrico	NOM-039-ECOL-1993 NOM-046-ECOL-1993
Compuestos orgánicos Volátiles (COV)	NOM-121-SEMARNAT-1997

ESTADO ACTUAL DE LOS SISTEMAS DE MONITOREO DE LA CALIDAD DEL AIRE EN MÉXICO

La NOM-156 regula el establecimiento y operación de sistemas de monitoreo de la calidad del aire. Esta norma especifica las condiciones mínimas y los sitios en donde los sistemas de monitoreo deben funcionar (campo de aplicación). En esta norma de cumplimiento obligatorio se establece que “los sistemas de monitoreo estarán conformados por una o más estaciones de muestreo y/o monitoreo, las cuales pueden ser fijas, semifijas o móviles”.

En el punto 2 de la NOM-156 se consignan cinco campos de aplicación para su cumplimiento, a saber:

1. Asentamientos humanos con más de quinientos mil habitantes;
2. Zonas metropolitanas;
3. Asentamientos humanos con emisiones superiores a veinte mil toneladas anuales de contaminantes criterio primarios a la atmósfera;
4. Conurbaciones;
5. Actividad industrial que por sus características se requiera del establecimiento de estaciones de monitoreo de calidad del aire y/o de muestreo de contaminantes atmosféricos.

A continuación, se muestra el cumplimiento de esta norma en cada uno de los cinco campos de aplicación mencionados anteriormente.

1. Asentamientos humanos con más de quinientos mil habitantes.

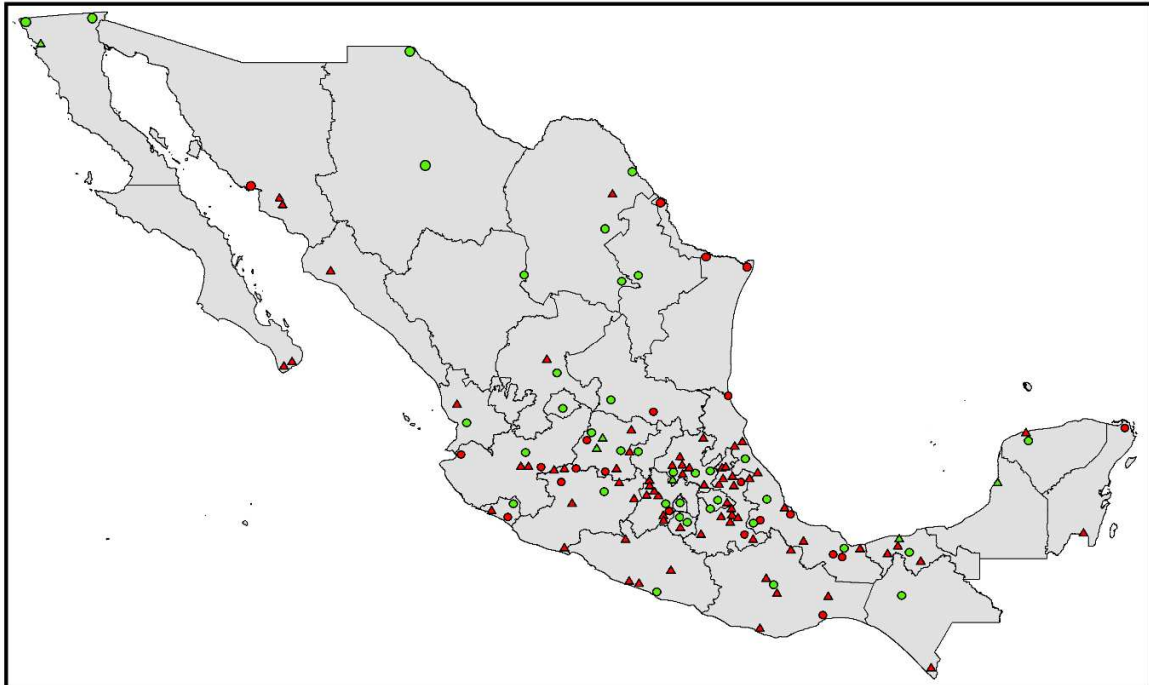
Victoria de Durango, la capital de Durango fue el único asentamiento con más de medio millón de habitantes que no se encuentra en el listado de las zonas metropolitanas ni en el de conurbaciones. Esta ciudad cumple con la NOM-156 al contar con un sistema de monitoreo de la calidad del aire formado por tres estaciones de monitoreo que reportan sus datos al SINAICA.

2. Zonas metropolitanas

En México se han delimitado 59 zonas metropolitanas constituidas por dos o más municipios con poblaciones mayores a 50,000 habitantes y por todos aquellos municipios que concentran un millón o más de habitantes (INEGI, 2017). En las zonas metropolitanas de México se concentran 66,729,005 habitantes que representa el 54.5% de la población (INEGI, 2017). La zona metropolitana de mayor tamaño es la zona metropolitana del valle de México cuya población alcanza 20 millones de habitantes (INEGI, 2016). Otras zonas metropolitanas de menor tamaño, pero de gran importancia, son las zonas metropolitanas de Guadalajara y Monterrey, cuyas poblaciones superan los cuatro millones cada una (INEGI, 2016). De las 59 zonas metropolitanas, 10 tienen poblaciones menores a 200 mil habitantes (Cuadro 4).

En el punto 10.5 de la NOM-156 se establece que los gobiernos de los estados, del Distrito Federal y de los Municipios deben remitir sus datos de calidad del aire al SINAICA. Sin embargo, el SINAICA no cuenta con los registros 42% de las zonas metropolitanas. En 8 de estas zonas la falta de datos se debe a que registran la información de la calidad del aire en estaciones de monitoreo manuales y quince de ellas no cuenta con sistemas de monitoreo de la calidad del aire, como es el caso de Cancún, Puerto Vallarta, Tehuacán y Veracruz (Figura 1).

Figura 1. Ubicación de las zonas metropolitanas (círculo) y de las zonas conurbadas (triángulo) en México. El color verde indica que cuenta con sistema de monitoreo de la calidad del aire. El color rojo indica que no cuenta con sistema de monitoreo.



La zona metropolitana del Valle de México posee el sistema de monitoreo de la calidad del aire más completo de la república, ya que cuenta con una red de 45 estaciones que cubren la mayor parte de su territorio. Las zonas metropolitanas de Guadalajara y Monterrey también cuentan con sistemas completos de monitoreo que cumplen con los requisitos normados con 10 estaciones de monitoreo cada una. Las zonas metropolitanas de Puebla-Tlaxcala y Valle de Toluca que tienen menos de tres millones de habitantes, cuentan con 6 y 7 estaciones de monitoreo respectivamente. A su vez en las 8 zonas metropolitanas con poblaciones entre uno y dos millones de habitantes los sistemas de monitoreo de la calidad del aire tienen entre 1 y 5 estaciones. De las 19 zonas metropolitanas que tienen entre medio y un millón de habitantes solo Veracruz y Cancún no tienen sistemas de monitoreo, mientras que parte las zonas metropolitanas de Tampico, Reynosa-Río Bravo y Matamoros no tienen datos de contaminación en el SINAICA porque cuentan con estaciones manuales. De las 17 zonas metropolitanas con poblaciones que van de entre doscientos mil y medio millón de habitantes, seis no cuentan sistemas de monitoreo de la calidad del aire mientras que, de las nueve zonas metropolitanas con menos de 200 mil habitantes, seis no tienen sistemas de monitoreo (Cuadro 4).

Cuadro 4. Zonas metropolitanas de la república mexicana, número de habitantes y el estado de la red de monitoreo de la calidad del aire en cada una de ellas (datos obtenidos del SINAICA; <http://sinaica.inecc.gob.mx/>).

Zona metropolitana	Estado	Población	Sistema monitoreo	Estaciones
Valle de México	Ciudad de México-Hidalgo-México	20,087,754	SI	45
Guadalajara	Jalisco	4,796,603	SI	10
Monterrey	Nuevo León	4,437,643	SI	10
Puebla-Tlaxcala	Puebla-Tlaxcala	2,876,940	SI	6
Valle de Toluca	México	2,012,672	SI	7
León	Guanajuato	1,967,501	SI	3
Tijuana	Baja California	1,751,430	SI	3
Juárez	Chihuahua	1,391,180	SI	4
La Laguna	Coahuila-Durango	1,283,835	SI	5
Querétaro	Querétaro	1,255,185	SI	5
San Luís Potosí	San Luís Potosí	1,133,571	SI	2
Mérida	Yucatán	1,058,764	SI	1
Aguascalientes	Aguascalientes	1,044,049	SI	4
Mexicali	Baja California	936 826	SI	4
Tampico	Tamaulipas-Veracruz	916,854	NO	–
Morelia	Michoacán	911,960	SI	3
Chihuahua	Chihuahua	902,823	SI	2
Cuernavaca	Morelos	899,563	SI	1
Acapulco	Guerrero	886,975	SI	1
Veracruz	Veracruz	880,444	NO	–
Villahermosa	Tabasco	823,213	SI	1
Saltillo	Coahuila	823,128	SI	1
Cancún	Quintana Roo	763,121	NO	–
Reynosa-Río Bravo	Tamaulipas	773,089	NO	–

Xalapa	Veracruz	719,591	SI	1
Tuxtla Gutiérrez	Chiapas	660,507	SI	1
Celaya	Guanajuato	731,667	SI	3
Oaxaca	Oaxaca	628,758	SI	2
Pachuca de Soto	Hidalgo	557,093	SI	2
Poza Rica	Veracruz	513,518	SI	1
Matamoros	Tamaulipas	520,367	NO	–
Tlaxcala-Apizaco	Tlaxcala	534,588	SI	
Cuatla	Morelos	475,441	SI	1
Tepic	Nayarit	429,351	SI	2
Orizaba	Veracruz	427,406	SI	2
Puerto Vallarta	Jalisco-Nayarit	425,890	NO	–
Nuevo Laredo	Tamaulipas	384033	NO	–
Coatzacoalcos	Veracruz	365,026	NO	–
Minatitlán	Veracruz	356,137	SI	1
Córdoba	Veracruz	347,647	NO	–
Colima-Villa de Álvarez	Colima	334,240	SI	1
Zacatecas-Guadalupe	Zacatecas	346,419	SI	1
Tehuacán	Puebla	344,603	NO	–
Monclova-Frontera	Coahuila	317,313	SI	1
Zamora-Jacona	Michoacán	265,952	NO	–
Tulancingo	Hidalgo	256,662	SI	1
La Piedad- Pénjamo	Guanajuato, Michoacán	254,272	NO	–
Tula	Hidalgo	225,219	SI	2
Guaymas	Sonora	203,430	NO	–
San Francisco del Rincón	Guanajuato	199,308	NO	–
Piedras Negras	Coahuila	180,734	SI	1
Tianguistenco	México	170,461	NO	–

Sto. Dom. Tehuantepec	Oaxaca	161,337	NO	–
Tecomán	Colima	141,421	NO	–
Ocotlán	Jalisco	141,375	NO	–
Río Verde-Ciudad Fdez.	San Luís Potosí	135,452	NO	–
Acayucan	Veracruz	127,495	NO	–
Teziutlán	Puebla	122,500	NO	–
Moroleón-Uriangato-	Guanajuato	108,669	NO	–

3. Asentamientos humanos con emisiones superiores a veinte mil toneladas anuales de contaminantes criterio primarios a la atmósfera

México está dividido en 32 entidades federativas, incluyendo a la Ciudad de México, que están compuestas por 2456 municipios y 16 delegaciones (INEGI 2017). Al revisar las emisiones de los contaminantes criterio primarios PM₁₀, PM_{2.5}, SO₂, CO y NO₂, se observó que las emisiones superan las 20,000 toneladas anuales en 352 poblaciones, el límite permitido por la NOM-156. En todos los casos el contaminante que más contribuye con este volumen de emisión es el CO que, por ejemplo, en Tijuana supera el millón y medio de toneladas. De estas 352 poblaciones, 124 cuentan con sistemas de monitoreo incluyendo ciudades como Guadalajara, Monterrey, Morelia y la ciudad de México y algunos poblados que hacen parte de algunas de las zonas metropolitanas. Las 228 poblaciones restantes no cuentan con sistemas de monitoreo propiamente establecidos y no reportan datos de calidad de aire al SINAICA, en estas 228 poblaciones de 28 de las entidades federativas viven algo más de 19 millones de habitantes que representa el 15.5% de la población. Veracruz, Michoacán y Jalisco son las entidades con mayor número de poblaciones que según la NOM-156 incumplen la obligación de tener sistemas de monitoreo de la calidad del aire y a reportar los datos al SINAICA (Figura 2). Las seis entidades federativas restantes, Aguascalientes, Baja California, la Ciudad de México, Hidalgo, Querétaro y Tlaxcala no tienen poblaciones

cuyas emisiones superan las 20 mil toneladas al año o, si rebasan este límite, cuentan con sistemas de monitoreo de la calidad del aire acorde a lo normado.

Figura 2. Número de habitantes desprovistos de sistemas de monitoreo de la calidad del aire en las diferentes entidades federativas de acuerdo a la NOM-156-SEMARNAT-2012.

4. Conurbaciones

Las conurbaciones son la “conformación urbana resultado de la continuidad física entre dos o más localidades geoestadísticas o centros urbanos, constituyendo una sola unidad urbana de por lo menos 15 mil habitantes. Pueden ser intermunicipales e interestatales cuando su población oscila entre 15 mil y 49 mil 999 habitantes e intramunicipales aun superando

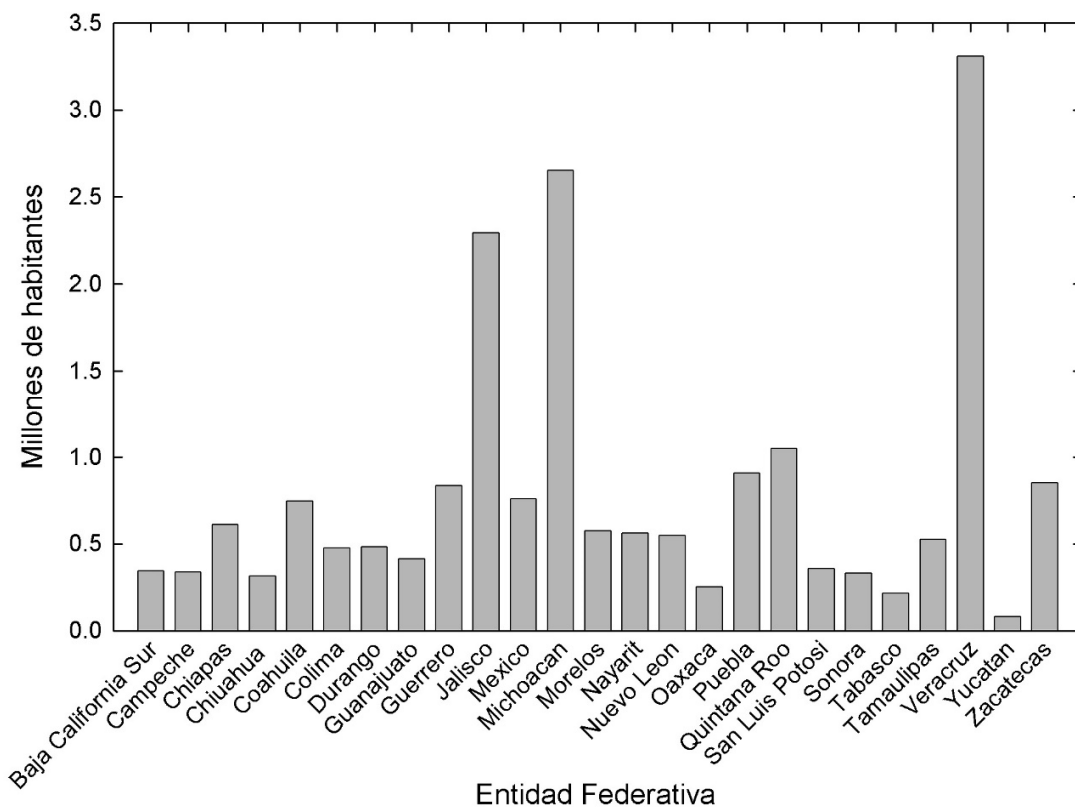


Figura 2. Número de habitantes desprovistos de sistemas de monitoreo de la calidad del aire en las diferentes entidades federativas de acuerdo con la NOM-156-SEMARNAT-2012.

este rango poblacional.” (CONAPO, 2012). En México existen 78 zonas conurbadas en las cuales habitan cerca de 5.5 millones de personas, lo que representa el 4.5% de la población. Únicamente en 6 zonas conurbadas se monitorea la calidad del aire, a saber, Ensenada, Campeche, Guanajuato, Irapuato, Tepeji y Paraíso en Tabasco. De estas, solo Ensenada, Irapuato y Tepeji reportan sus datos al SINAICA. Las 72 zonas conurbadas restantes no cuentan con sistemas de monitoreo de la calidad del aire. Con esto queda evidenciado el incumplimiento de lo establecido en los puntos 2 y 10.5. de la NOM-156.

5. Actividad industrial que por sus características se requiera del establecimiento de estaciones de monitoreo de calidad del aire y/o de muestreo de contaminantes atmosféricos.

La NOM-156 no especifica las características particulares que deben tener las actividades industriales sujetas a la implementación de un sistema de monitoreo de la calidad del aire. Sin embargo, en el punto 6 se hace claridad acerca de que se deben hacer estudios preliminares para conocer el estado de la calidad del aire en sitios que puedan ser susceptibles de contaminación para determinar la necesidad de implementar sistemas de monitoreo, el tipo de sistemas de acuerdo a las condiciones de cada sitio y establecer los objetivos del monitoreo.

CONCLUSIONES

México es un país que cuenta con la normatividad necesaria para asegurar que sus habitantes conozcan el estado de la calidad del aire del lugar que habitan. Sin embargo, gran número de poblaciones no cuentan con sistemas de monitoreo ni reportan sus datos al SINAICA pese a que según lo normado así lo requieren. Al menos 20 millones de personas no tienen acceso a la información concerniente a la calidad del aire de su región. Se debe llevar a cabo una revisión de los centros urbanos en incumplimiento de la NOM-156 para identificar las causas y determinar acciones correctivas pertinentes. Se debe promover un programa federal que incentive y apoye la implementación de sistemas de monitoreo de la calidad del aire, pero que también imponga sanciones a las poblaciones que incumplan con lo normado.

La emisión de contaminantes cuyo monitoreo no está normado o se incumple, además de tener efectos adversos en la salud pública, causa daños al ambiente y puede contribuir con el cambio climático, en discordancia con lo previsto en la Ley General de Cambio Climático. Se recomienda incluir en la normatividad de monitoreo de la calidad de aire contaminantes tales como las especies químicas de óxidos de nitrógeno que están involucradas en la formación de esmog en las ciudades y son de gran importancia en salud pública. Se recomienda incluir en la normatividad el monitoreo de compuestos orgánicos volátiles ya que están involucrados en la formación del smog de las ciudades y del ozono troposférico, estos compuestos también son de importancia en salud pública. Por su parte, en las zonas donde se lleva a cabo la agricultura se debe considerar el monitoreo de la calidad del aire a fin de conocer el impacto que los contaminantes emitidos por estos sistemas productivos tienen en la calidad del aire local y regional. Pues cabe recordar que la NOM-156 se concentra en zonas urbanas y hasta 40% de la población mexicana habita en zonas rurales.

Una alternativa que está siendo probada desde algunos años es la implementación de sistemas de monitoreo con organismos biomonitores que permiten conocer el estado de contaminación, esta podría ser una alternativa viable en lugares donde la calidad del aire no se monitorea adecuadamente. De considerarse los fallos aquí encontrados y tener en cuenta las recomendaciones será posible asegurar que la población, así como los tomadores de decisiones tengan conocimiento de la calidad del aire de su región y puedan promover acciones para mejorar sus condiciones de vida.

Reconocimientos

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Apéndice 1. Normas oficiales mexicanas en materia de contaminación atmosférica, emisiones provenientes de fuentes móviles.

Norma	Materia
NOM-041-SEMARNAT-2015	Establece los niveles máximos permisibles de emisión de gases contaminantes provenientes del escape de los vehículos automotores en circulación que usan gasolina como combustible.
NOM-042-SEMARNAT-2003	Que establece los límites máximos permisibles de emisión de hidrocarburos totales o no metano, monóxido de carbono, óxidos de nitrógeno y partículas provenientes del escape de los vehículos automotores nuevos cuyo peso bruto vehicular no exceda los 3,857 kilogramos, que usan gasolina, gas licuado de petróleo, gas natural y diesel, así como de las emisiones de hidrocarburos evaporativos provenientes del sistema de combustible de dichos vehículos.
NOM-044-SEMARNAT-2006	Establece los límites máximos permisibles de emisión de hidrocarburos totales, hidrocarburos no metano, monóxido de carbono, óxidos de nitrógeno, partículas y opacidad de humo provenientes del escape de motores nuevos que usan diesel como combustible y que se utilizarán para la propulsión de vehículos automotores nuevos con peso bruto vehicular mayor de 3,857 kilogramos, así como para unidades nuevas con peso bruto vehicular mayor a 3,857 kilogramos equipadas con este tipo de motores.
NOM-045-SEMARNAT-2006	Protección ambiental. Vehículos en circulación que usan diesel como combustible. Límites máximos permisibles de opacidad, procedimiento de prueba y características técnicas del equipo de medición.
NOM-047-SEMARNAT-1999	Establece las características del equipo y el procedimiento de medición para la verificación de los niveles de emisión de contaminantes, provenientes de los vehículos automotores en circulación que usan gasolina, gas licuado de petróleo, gas natural u otros combustibles alternos
NOM-048-ECOL-1993	Establece los niveles máximos permisibles de emisión de hidrocarburos, monóxido de carbono y humo, provenientes del escape de las motocicletas en circulación que utilizan gasolina o mezcla de gasolina-aceite como combustible.
NOM-049-ECOL-1993	Establece las características del equipo y el procedimiento de medición, para la verificación de los niveles de emisión de gases contaminantes, provenientes de las motocicletas en circulación que usan gasolina o mezcla de gasolina-aceite como combustible.
NOM-050-ECOL-1993	Establece los niveles máximos permisibles de emisión de gases contaminantes provenientes del escape de los vehículos automotores en circulación que usan gas licuado de petróleo, gas natural u otros combustibles alternos como combustible.
NOM-076-SEMARNAT-2012	Establece los niveles máximos permisibles de emisión de hidrocarburos no quemados, monóxido de carbono y óxidos de nitrógeno provenientes del

	escape, así como de hidrocarburos evaporativos provenientes del sistema de combustible, que usan gasolina, gas licuado de petróleo, gas natural y otros combustibles alternos y que se utilizarán para la propulsión de vehículos automotores con peso bruto vehicular mayor de 3,857 kilogramos nuevos en planta.
NOM-077-ECOL-1995	Establece el procedimiento de medición para la verificación de los niveles de emisión de la opacidad del humo proveniente del escape de los vehículos automotores en circulación que usan diesel como combustible.

Apéndice 2. Normas oficiales mexicanas en materia de contaminación atmosférica, emisiones provenientes de fuentes fijas.

NORMA	MATERIA
NOM-039-ECOL-1993	Establece los niveles máximos permisibles de emisión a la atmósfera de bióxido y trióxido de azufre y neblinas de ácido sulfúrico, en plantas productoras de ácido sulfúrico.
NOM-040-ECOL-2002	Protección ambiental-Fabricación de cemento hidráulico-Niveles máximos permisibles de emisión a la atmósfera.
NOM-043-ECOL-1993	Establece los niveles máximos permisibles de emisión a la atmósfera de partículas sólidas provenientes de fuentes fijas.
NOM-046-ECOL-1993	Establece los niveles máximos permisibles de emisión a la atmósfera de bióxido de azufre, neblinas de trióxido de azufre y ácido sulfúrico, provenientes de procesos de producción de ácido dodecibencensulfónico en fuentes fijas.
NOM-051-ECOL-1993	Establece el nivel máximo permisible en peso de azufre, en el combustible líquido, gasóleo industrial que se consuma por las fuentes fijas en la zona metropolitana de la Ciudad de México.
NOM-085-SEMARNAT-2011	Contaminación atmosférica-Niveles máximos permisibles de emisión de los equipos de combustión de calentamiento indirecto y su medición.
NOM-086-SEMARNAT-SENER-SCFI-2005	Especificaciones de los combustibles fósiles para la protección ambiental.
NOM-097-ECOL-1995	Establece los límites máximos permisibles de emisión a la atmósfera de material particulado y óxidos de nitrógeno en los procesos de fabricación de vidrio en el país. (1a. aclaración 01-julio-1996. y 2a. aclaración 16-octubre-1996).
NOM-098-SEMARNAT-2002	Incineración de residuos, especificaciones de operación y límites de emisión de contaminantes.

NOM-105-ECOL-1996	Establece los niveles máximos permisibles de emisiones a la atmósfera de partículas sólidas totales y compuestos de azufre reducido total provenientes de los procesos de recuperación de químicos de las plantas de fabricación de celulosa.
NOM-137-SEMARNAT-2013	Contaminación atmosférica. Complejos procesadores de gas. Control de emisiones de compuestos de azufre.
NOM-148-SEMARNAT-2006	Contaminación atmosférica. Recuperación de azufre proveniente de los procesos de refinación del petróleo.
NOM-166-SEMARNAT-2014	Control de emisiones atmosféricas en la fundición secundaria de plomo.
NOM-121-SEMARNAT-1997	Establece los límites máximos permisibles de emisión a la atmósfera de compuestos orgánicos volátiles (COV) provenientes de las operaciones de recubrimiento de carrocerías nuevas en planta de automóviles, unidades de uso múltiple, de pasajeros y utilitarios; carga y camiones ligeros, así como el método para calcular sus emisiones.

Apéndice 3. Normas oficiales mexicanas en materia de contaminación atmosférica, monitoreo ambiental y salud de la población.

Norma	Descripción
NOM-020-SSA1-2014	Valor límite permisible para la concentración de ozono (O ₃) en el aire ambiente y criterios para su evaluación.
NOM-021-SSA1-1993	Criterio para evaluar la calidad del aire ambiente con respecto al monóxido de carbono (CO). Valor permisible para la concentración de monóxido de carbono (CO) en el aire ambiente.
NOM-022-SSA1-2010	Criterio para evaluar la calidad del aire ambiente, con respecto al dióxido de azufre (SO ₂). Valor normado para la concentración de dióxido de azufre (SO ₂) en el aire ambiente.
NOM-023-SSA1-1993	Criterio para evaluar la calidad del aire ambiente, con respecto al bióxido de nitrógeno (NO ₂). valor normado para la concentración de bióxido de nitrógeno (NO ₂) en el aire ambiente.
NOM-025-SSA1-2014	Valores límite permisibles para la concentración de partículas suspendidas PM ₁₀ y PM _{2.5} en el aire ambiente y criterios para su evaluación.
NOM 026-SSA1-1993.	Criterio para evaluar la calidad del aire ambiente, con respecto al plomo (Pb). Valor normado para la concentración de plomo (Pb) en el aire ambiente.
NOM-034-SEMARNAT-1993	Establece los métodos de medición para determinar la concentración de monóxido de carbono en el aire ambiente y los procedimientos para la calibración de los equipos de medición
NOM-035-SEMARNAT-1993	Establece los métodos de medición para determinar la concentración de partículas suspendidas totales en el aire ambiente y el procedimiento para la calibración de los equipos de medición.
NOM-036-ECOL-1993	Establece los métodos de medición para determinar la concentración de ozono en el aire ambiente y los procedimientos para la calibración de los equipos de medición.
NOM-037-SEMARNAT-1993	Establece los métodos de medición para determinar la concentración de bióxido de nitrógeno en el aire ambiente y los procedimientos para la calibración de los equipos de medición.
NOM-038-SEMARNAT-1993	Establece los métodos de medición para determinar la concentración de bióxido de azufre en el aire ambiente y los procedimientos para la calibración de los equipos de medición.
NOM-156-SEMARNAT-2012	Establecimiento y operación de sistemas de monitoreo de la calidad del aire.

CAPÍTULO VII

Artículo:

Acumulación nocturna de acidez titulable por *Tillandsia makoyana* (Bromeliaceae), epífita de la selva baja caducifolia

Acumulación nocturna de acidez titulable por *Tillandsia makoyana* (Bromeliaceae), epífita de la selva baja caducifolia

Nocturnal accumulation of titratable acidity in *Tillandsia makoyana* (Bromeliaceae), epiphytic bromeliad of the tropical dry forest

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Resumen. Se estudió la bromelia epífita *Tillandsia makoyana* para evaluar la contribución de los factores ambientales agua, temperatura y luz en la expresión del metabolismo ácido de las Crasuláceas. En particular, se determinaron la acumulación nocturna de ácido málico, la temperatura máxima de la hoja en respuesta al riego y la cantidad de luz incidente. Los tratamientos de riego y luz directa determinaron la mayor acumulación nocturna de acidez titulable ($24,52 \pm 0,34$ mmol H⁺/m²), mientras que aquella en las plantas sin riego y bajo sombra fue 59% menor. Los resultados ilustraron una respuesta rápida de la actividad fotosintética a la disponibilidad de agua en *Tillandsia makoyana*, especie originaria de un ambiente donde este factor ambiental es muy fluctuante.

Palabras clave: Chamela; Epífitas; Metabolismo ácido de las crasuláceas; Relaciones hídricas; Sombra.

Abstract. The epiphytic bromeliad *Tillandsia makoyana* was studied to assess the contribution of the environmental factors, water, temperature, and light, to the expression of the Crassulacean acid metabolism. In particular, nocturnal titratable acidity accumulation and maximum leaf temperature were measured in response to watering and incident light. Plants that were watered and exposed to direct sunlight had the highest nocturnal accumulation of titratable acidity (25.52 ± 0.34 H⁺/m²), while those without irrigation and shaded showed a 59% reduction in their titratable acidity. Our results illustrated a rapid response of the photosynthetic activity to water availability in *Tillandsia makoyana*, a native species from an ecosystem where this environmental factor fluctuates greatly.

Keywords: Chamela; Crassulacean acid metabolism; Epiphytes; Shade; Water relations.

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INTRODUCCIÓN

Las plantas epífitas poseen adaptaciones morfológicas, anatómicas y fisiológicas que les permiten resistir las condiciones generalmente secas del dosel. Estas adaptaciones incluyen la fotosíntesis mediada por el metabolismo ácido de las crasuláceas (CAM), la succulencia en hojas y la presencia de tricomas epidérmicos (Andrade et al., 2004; Reyes-García y Griffiths, 2009). Por ejemplo, en bromelias epífitas CAM, el potencial hídrico foliar disminuye durante la noche por la acumulación de ácidos orgánicos, lo que incrementa su capacidad de absorber el rocío matinal que es canalizado hacia los estomas por los tricomas foliares (Griffiths, 1988; Andrade, 2003). Se ha estimado que el 57% de las plantas epífitas presentan CAM y que tienden a ser más abundantes que las epífitas C_3 en los sitios más expuestos del dosel y en los bosques más secos (Benzing, 1987; Zotz, 2004).

Por su parte, los distintos ambientes luminosos que se presentan en el dosel han dado origen a una estratificación de las especies epífitas (Andrade et al., 2004; Lüttge, 2004). Por ejemplo, las especies con hojas más pequeñas pero con elevada succulencia y superficies más glaucas son más frecuentes en los estratos más expuestos. En contraste, las hojas de las especies que habitan las partes más sombreadas del dosel tienden a presentar láminas con una mayor superficie pero menor grosor y, en consecuencia, un menor grado de succulencia, además de una concentración mayor de clorofila que sus contrapartes de hábito más expuesto.

Para conocer la respuesta a la luz y a la disponibilidad de agua en la expresión del CAM por la epífita *Tillandsia makoyana* (Bromeliaceae), especie de la selva baja caducifolia, se determinaron su acumulación nocturna de ácido málico y la temperatura máxima foliar en función de la disponibilidad de estos dos factores ambientales.

MATERIALES Y MÉTODOS

El estudio se realizó en la selva baja caducifolia de la Estación de Biología Chamela (19° 30' N, 105° 03' O) de la Universidad Nacional Autónoma de México, la cual forma parte de la Reserva de la Biósfera Chamela-Cuixmala, donde cerca del 80% de la precipitación anual (746 mm) ocurre de julio a octubre y la temperatura media es de 25 °C (Bullock, 1986; datos climáticos de la Estación).

El 7 de noviembre de 2011 se obtuvieron individuos de *Tillandsia makoyana* Baker (*sensu* Espejo-Serna et al., 2004) con buena vitalidad, que habían sido derribados por el huracán Jova el 12 de octubre de 2011 (Bravo y Hernández, 2011). Las plantas, de aproximadamente 30 cm de alto y 30 cm de diámetro, fueron colocadas en superficies firmes de concreto y sostenidas con rocas pequeñas de tal manera que el eje de cada roseta tuviera una posición ortogonal al piso. En esa posición, las plantas fueron aclimatadas durante dos días a la luz directa

del sol ($805 \pm 2 \mu\text{mol}/\text{m}^2/\text{s}$, medido a medio día del 7 al 10 de noviembre de 2011, con un cuantómetro LI-190S; LI-COR Lincoln, Nebraska, EEUU) o a la sombra proyectada por un edificio aledaño y por árboles circundantes ($210 \pm 2 \mu\text{mol}/\text{m}^2/\text{s}$). Al mismo tiempo se estableció un tratamiento de riego (200 mL de agua corriente por planta depositados cuidadosamente en cada roseta con un movimiento en espiral de tal manera que todo el líquido quedara contenido en el tanque de la planta) para la mitad de las plantas de cada grupo.

La actividad fotosintética de las plantas se estimó por medio de la acumulación nocturna de acidez titulable (H^+ ; Osmond et al., 1994). El 10 de noviembre de 2011 a las 18:00 y el 11 de noviembre de 2011 a las 6:00 h se tomaron muestras de hoja de cada una de las plantas con un sacabocados (área de 1,5 cm²). Las muestras fueron puestas en etanol (80% v/v) y hervidas en 10 mL de agua sin dejar que ésta se evaporara por completo, antes de ser homogeneizadas en un mortero y aforadas a 50 mL con agua destilada. La solución del macerado se tituló con una solución acuosa de NaOH (0,01 N) hasta alcanzar pH neutro. Por su parte, la temperatura superficial de las plantas se midió a medio día durante el estudio con un termómetro infrarrojo OMEGAETTE OS542 (Omega Engineering, Stamford, CT, EEUU).

El desempeño de *T. makoyana* bajo los distintos tratamientos de luz y riego fue comparado mediante un análisis de varianza de dos vías, seguido de pruebas pareadas de Tukey ($P < 0,05$) usando Sigmaplot 12 (Systat Software, Richmond, California, EEUU). Los datos se muestran como media \pm 1 E.E. ($n = 5$ plantas por tratamiento).

RESULTADOS

La acumulación nocturna de ácidos orgánicos por *Tillandsia makoyana* respondió a la interacción de la luz y el riego (Tabla 1). Las plantas expuestas a insolación directa y que recibieron riego presentaron la mayor acumulación nocturna de ácidos orgánicos ($24,52 \pm 0,34 \text{ mmol H}^+/\text{m}^2$; Fig. 1). La acumulación nocturna más baja de ácidos orgánicos (59% menor

Tabla 1. ANOVA de dos vías de la respuesta de la acumulación nocturna de acidez titulable y de la temperatura superficial en *Tillandsia makoyana* bajo la sombra o expuestas a la luz directa del sol y sometidas a dos condiciones de riego.

Table 1. Two-way ANOVA of the response to the nocturnal accumulation of titratable acidity and superficial temperature in *Tillandsia makoyana* under the shade or exposed to direct solar radiation after exposure to two irrigation conditions.

	G.L.	Acidez titulable		Temperatura superficial	
		F	P	F	P
Luz	2	157,971	< 0,001	642359	< 0,001
Riego	1	912,013	< 0,001	19,827	< 0,001
Luz \times Riego	2	62,754	< 0,001	1,132	0,350

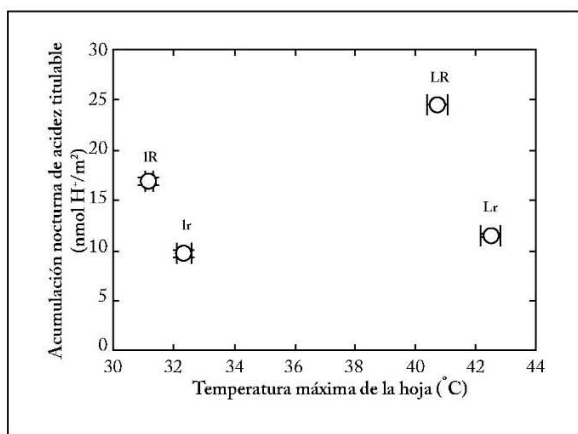


Fig. 1. Acumulación nocturna de acidez titulable y temperatura máxima superficial de *Tillandsia makoyana* en respuesta al riego y a la exposición a la luz solar. Los datos se muestran como promedio \pm E.E. ($n = 5$ individuos) para plantas que recibieron insolated directa (L) o que estaban bajo sombra (l) y que fueron regadas (R) o tuvieron el riego suspendido (r).

Fig. 1. Nocturnal accumulation of titratable acidity and maximum superficial temperature of *Tillandsia makoyana* in response to irrigation and exposure to solar radiation. Data are the means \pm S.E. ($n = 5$ individuals) for plants that received direct solar radiation (L), or were shaded (l), and were either irrigated (R) or not (r).

que la más alta) fue observada en las plantas mantenidas a la sombra y sin riego (Fig. 1).

La temperatura superficial de las hojas también respondió a la exposición a la luz y al riego (Tabla 1). En este caso la mayor exposición y la falta de riego resultaron en la temperatura más alta ($42,2 \pm 0,3$ °C), mientras que la menor temperatura ($31,2 \pm 0,1$ °C), se obtuvo en las plantas regadas bajo sombra (Fig. 1).

DISCUSIÓN

Tillandsia makoyana presenta su máxima acumulación nocturna de acidez titulable durante la estación de lluvias de julio a octubre (Reyes-García et al., 2008, 2011; Reyes-García y Griffiths, 2009). Aquí se observó, de manera similar, que los individuos expuestos a riego presentaron una mayor acumulación de ácidos orgánicos que las plantas sin riego. Sin embargo, contrastó el hecho de que las plantas expuestas a la insolated directa presentaron un mejor desempeño que aquellas bajo sombra, debido a que un aumento en el índice de área foliar en el dosel durante la época de lluvias causa que la disponibilidad de luz en el mismo sea relativamente baja en Chamela, pudiendo alcanzar solo entre 20 y 30% del flujo de fotones fotosintéticos máximo (Nobel y de la Barrera, 2004).

Además de que la precipitación es marcadamente estacional, la disponibilidad de agua en el dosel ocurre en pulsos (Murphy y Lugo, 1986; Andrade y Nobel, 1997). Las plantas

epífitas son capaces de sobrellevar esta irregularidad en la disponibilidad de agua disminuyendo rápidamente su actividad fotosintética en respuesta a la sequía. Esto se puede deber al cierre de los estomas mediado por el ácido giberélico (Acevedo et al., 1983; Stancantoa et al., 2001; Graham y Andrade, 2004). Paralelamente, estas plantas son capaces de recuperar rápidamente su función fisiológica cuando el agua vuelve a estar disponible. Por ejemplo, el cactus hemiepífito *H. undatus* expuesto a una sequía de dos semanas recupera su tasa máxima de intercambio gaseoso ocho días después de reiniciar el riego (Nobel y de la Barrera, 2002).

La temperatura superficial de las hojas medida al medio día fue menor en las plantas con riego que en aquellas sin riego, lo que podría atribuirse a una pérdida de calor por transpiración diurna en esta especie CAM inducida por el riego (Hartsock y Nobel, 1976; Andrade et al., 2007). Sin embargo, *T. makoyana* se reporta como una especie CAM estricta que no realiza intercambio gaseoso durante el día aún bajo condiciones de buen riego (Mooney et al., 1989; Reyes-García et al., 2008). En este caso, la diferencia de temperatura observada podría deberse a pérdida de calor latente durante la evaporación del agua contenida en el tanque de las plantas.

En plantas CAM, la acumulación nocturna de acidez titulable (ΔH^+) responde directamente al flujo de fotones incidente sobre las plantas durante el día anterior (Andrade et al., 2007). En este estudio el flujo de fotones no influyó de manera sustancial en ΔH^+ , pues un aumento cercano al 400% en dicho flujo apenas produjo un incremento en la acumulación ΔH^+ de 33% en plantas con riego y de 16% en plantas con sombra. En contraste, para *T. usneoides* la máxima ΔH^+ ocurre bajo 125 $\mu\text{mol}/\text{m}^2/\text{s}$ y se inhibe sustancialmente bajo flujos de fotones mayores o menores (Martin et al., 1986). La amplitud del flujo de fotones adecuada para la fotosíntesis en *T. makoyana* probablemente es el resultado de su adaptación a los niveles de luz presentes en la parte media del dosel donde habita (Reyes-García y Griffiths, 2009).

En este estudio se confirmó que las plantas epífitas, como *Tillandsia makoyana*, responden de manera muy rápida a la disponibilidad de agua. Futuras investigaciones deberían considerar a otras especies, como *T. rothii* que crece en las partes más externas del dosel o *Achmea bracteata* de hábito terrestre. Esto permitiría conocer las diferentes adaptaciones que han desarrollado estas especies del bosque seco.

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CAPÍTULO VIII

DISCUSIÓN GENERAL

En este trabajo doctoral se determinó la variación isotópica que presenta una orquídea endémica de México creciendo en dos sitios con actividades humanas contrastantes. La fuente de nitrógeno de *Laelia speciosa* en el campo fue casi en su totalidad el aire limpio, situación que se mantuvo durante al menos 10 años antes al momento del estudio, esto de acuerdo a los $\delta^{15}\text{N}$ de las series de pseudobulbos. Por su parte las plantas que fueron trasladadas del campo a la ciudad obtuvieron su nitrógeno principalmente del aire de este centro urbano, dicho aire contiene trazas de contaminación por actividad industrial y vehicular. Adicionalmente, en este trabajo se pudo determinar cómo los nutrientes y particularmente el nitrógeno es reasignado de los pseudobulbos más viejos a aquellos en formación. En este trabajo doctoral también se investigó el efecto de dosis crecientes de depósito de nitrógeno sobre la orquídea *Laelia speciosa* y se encontró que las orquídeas sometidas a dosis menores a o iguales a $20 \text{ kg de N ha}^{-1} \text{ año}^{-1}$ les sirve como fertilizante ya que incrementaron el número de órganos producidos y el contenido de clorofila, por el contrario, las dosis mayores o iguales a 40 y $80 \text{ kg de N ha}^{-1} \text{ año}^{-1}$ tuvieron efectos tóxicos sobre esta orquídea.

Laelia speciosa mostró ser especialmente sensible a los cambios en la disponibilidad de nitrógeno dada su fuerte dependencia de fuentes atmosféricas para la obtención de nutrientes en el ambiente epifito. Esto se evidencia cuando al aumentar la dosis de nitrógeno sobre los $20 \text{ N ha}^{-1} \text{ año}^{-1}$ la fisiología de la orquídea se vio afectada, de manera similar a lo que se observa en los musgos *Braunia secunda*, *Pseudoscleropodium purum*, *Rhytidiadelphus squarrosus*, *Thuidium delicatulum*, el pino *Pinus densiflora* y el abeto *Cunninghamia lanceolata* (Nakaji et al. 2001; Arróniz-Crespo et al. 2008; Ying-Chun et al. 2010). En contraste dos especies de bromelias epifitas *Tillandsia recurvata* y *T. usneoides* no se detecta ningún efecto aun cuando la dosis alcanza los $40 \text{ kg de N año}^{-1}$ (Barrios 2009), ni el cedro japonés *Cryptomeria japonica* al que aplicaron dosis de hasta $340 \text{ kg de N año}^{-1}$ (Nakaji et al. 2001).

Los resultados observados en estos dos capítulos (3 y 4) sobre la respuesta fisiológica y la variación en las fuentes de nitrógeno en dos lugares con actividades humanas contrastantes, conllevaron al desarrollo del capítulo 5 en el cual se exploró la viabilidad en el uso de diferentes organismos biomonitores para la determinación de la fuente y la tasa de depósito de nitrógeno atmosférico a mayor escala. Para ello, se seleccionaron 4 especies de

tres tipos de organismos cuya nutrición se basa principalmente en fuentes atmosféricas. También, se escogió una de las zonas con mayor población del planeta, la Ciudad de México y el Valle que la contiene. La ciudad de México cuenta con una serie de estaciones de monitoreo de la calidad del aire y del depósito de nitrógeno distribuidas en gran parte de la ciudad y tiene una base de datos de libre acceso. Al comparar los datos obtenidos de la red de monitoreo y superponer lo encontrado en los diferentes biomonitores se observa claramente la influencia del depósito de nitrógeno atmosférico sobre estos. El principal aporte que obtuvimos de este estudio fue que, de los tres tipos de organismos, el líquen mostró la respuesta menos clara al depósito atmosférico. A su vez, el musgo *Grimmia* sp. respondió directamente al depósito de nitrógeno húmedo de la Ciudad de México y en menor medida a la concentración de NO_x. La bromelia epífita *Tillandsia recurvata*, mostro una relación directa con la concentración de NO_x debido a su fotosíntesis tipo CAM. Si bien los bioindicadores no son un estimador exacto de la magnitud del depósito atmosférico, dan una idea de la cantidad y la fuente del nitrógeno que reciben los ecosistemas, por lo que son de gran utilidad en identificar áreas sujetas a la contaminación por nitrógeno atmosférico. En combinación los valores isotópicos y el contenido de nitrógeno en los tejidos de plantas que dependen de la atmosfera como única fuente de nutrientes son buenos indicadores del depósito de nitrógeno atmosférico y alertar sobre las primeras etapas de saturación que podrían producir daño a los ecosistemas expuestos al incremento en el depósito atmosférico. Adicionalmente, el bajo costo que supone el uso de esta metodología puede contribuir al monitoreo de grandes extensiones de territorio.

Del trabajo llevado a cabo en la Ciudad de México se derivó el capítulo 6, en el que se analizó la legislación mexicana con relación al monitoreo de la calidad del aire. En particular, se analizó el cumplimiento de la NOM-156-SEMARNAT-2012. Esta norma dicta que aquellos centros urbanos que cumplan ciertas características deben tener sistemas de monitoreo de la calidad del aire. Un análisis de su cumplimiento reveló que quince zonas metropolitanas y 72 zonas conurbadas no cumplen con lo normado. También, encontramos que 352 centros urbanos tienen emisiones de contaminantes criterio superiores a las 20 mil toneladas anuales permitidas, de las cuales 228 no cuentan con sistemas de monitoreo de la calidad del aire. Por lo anterior, al menos 20 millones de personas de todo México no cuentan con cubrimiento de sistemas de monitoreo debido a que viven en poblaciones que según la

NOM-156 están obligadas a monitorear la calidad del aire y no lo hacen. Se recomienda realizar una revisión de los centros urbanos en incumplimiento para determinar las causas y acciones a tomar, así como considerar ampliar el conjunto de contaminantes monitoreados de importancia en salud pública y ambiental.

Finalmente, con el objetivo de determinar la respuesta de una bromelia atmosférica a los cambios en la provisión de agua. Se estudió la bromelia epífita *Tillandsia makoyana* para evaluar la contribución de los factores ambientales agua, temperatura y luz en la expresión del metabolismo ácido de las Crasuláceas. En particular, se determinaron la acumulación nocturna de ácido málico, la temperatura máxima de la hoja en respuesta al riego y la cantidad de luz incidente. Los tratamientos de riego y luz directa determinaron la mayor acumulación nocturna de acidez titulable ($24,52 \pm 0,34$ mmol H⁺/m²), mientras que aquella en las plantas sin riego y bajo sombra fue 59% menor. Los resultados ilustraron una respuesta rápida de la actividad fotosintética a la disponibilidad de agua en *T. makoyana*, especie originaria de un ambiente donde este factor ambiental es muy fluctuante.

Esta tesis doctoral contribuyó al entendimiento sobre el uso de organismo de nutrición atmosférica como biomonitores del depósito de nitrógeno atmosférico y sus posibles implicaciones para la formulación de políticas públicas en pro del conocimiento sobre el estado de los ecosistemas y otros ambientes con respecto a la contaminación atmosférica.

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APÉNDICE

Artículo:

In vitro germination and development of two endangered endemic Colombian orchids *Cattleya mendelii* and *Cattleya quadricolor*

In vitro* germination and development of two endangered endemic Colombian orchids *Cattleya mendelii* and *Cattleya quadricolor

Germinación y desarrollo *in vitro* de dos orquídeas amenazadas endémicas de Colombia, *Cattleya mendelii* y *Cattleya quadricolor*

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ABSTRACT

Cattleya mendelii and *C. quadricolor* are endemic orchids from Colombia, which have been subjected to extraction from their natural environments for commercial purposes, becoming critically endangered. However, there is no sustainable management practice for the conservation of these species. The aim of this study was to establish a protocol of *in vitro* culture for both species. The effects of different combinations of GA₃ and NAA amending MS and KC culture media were assessed for the *in vitro* germination and plant growth. Plant development was assessed monthly over 120 days. A higher percentage of explant production for both species was observed with MS than KC. The higher GA₃ proportion of 1.5 µM resulted in enhanced germination, while the higher NAA concentration of 1.5 µM favored later stages of plant development. For instance, the number of roots and the length of roots and shoots were higher under incubation on MS than under KC for both species. *In vitro* germination for *C. mendelii* and for *C. quadricolor* was successful for their propagation and could be useful in future conservation programs for these species.

KEYWORDS: Biotechnology, conservation, culture media, growth regulators, plant development.

RESUMEN

Cattleya mendelii y *C. quadricolor* son dos orquídeas endémicas de Colombia que han sido sujetas a extracción de sus ambientes naturales para la venta, esto ha conducido a estas plantas a estar críticamente amenazadas. En la actualidad, no existe una práctica sustentable para el manejo de estas especies. El objetivo de este estudio fue establecer un protocolo de cultivo *in vitro* para ambas especies. El efecto de diferentes combinaciones de medios de cultivo MS y KC modificados con AG₃ y ANA fueron evaluados para la germinación y desarrollo *in vitro*. El desarrollo fue evaluado cada 30 días, durante 120 días. La mayor producción de explantes para ambas especies fue observada con MS. Mayor proporción de AG₃ de 1.5 µM resultó en el aumento de la germinación, asimismo mayores concentraciones de ANA de 1.5 µM favorecen los estadios tardíos del desarrollo vegetal *in vitro*. El número y la longitud de los brotes y las raíces fueron mayores cuando las plantas de ambas especies se cultivaron en medio MS. La germinación y desarrollo *in vitro* de *C. mendelii* and *C. quadricolor* en este estudio fue una aproximación exitosa para la propagación y puede ser considerada para programas de conservación de estas orquídeas.

PALABRAS CLAVE: Biotecnología, conservación, desarrollo vegetal, medios de cultivo, reguladores del crecimiento vegetal.

INTRODUCTION

The genus *Cattleya* (Orchidaceae), native to Central and South America, occurs along mountain ranges, dry forests, and the transition to wet and cloudy hillsides and canyons,

mainly on trees and rocks (Calderón 2007). *Cattleya mendelii* Dombroin and *C. quadricolor* Lindl. are endemic to Colombia and have been traditionally kept in home gardens owing to their beautiful flowers. Additionally, the over-extraction from their habitat for sale at local and international markets has

led to an 80% reduction of their wild populations over the last century. As a result, these orchids have been recognized by the International Union for Conservation of Nature as a critically endangered species (Calderón 2007).

To date, there is no a sustainable management practice for these species of endangered orchids. In this respect, the *in vitro* propagation of vascular plants is a useful method that can be utilized for commercial or for conservation purposes as an alternative that might help in decreasing the extractive pressure on natural populations of endangered plants (Rubluo *et al.* 1993, Arditti & Krikorian 1996, Buyun *et al.* 2004, Lo *et al.* 2004, Santos-Hernández *et al.* 2005, Ávila-Díaz *et al.* 2009). However, the *in vitro* propagation of orchids requires a species-specific method for massive and rapid production of these plants (Arditti 1977, Colli & Kerbauy 1993, Shimura & Koda 2003).

Not only the form of nitrogen provided to the culture media but also the presence and concentration of some growth regulators can affect the rate of emergence and development of orchid seeds during *in vitro* culture (Ichihashi 1992, Chen *et al.* 2000, Park *et al.* 2002, Jawan *et al.* 2010). In consequence, medium optimization, which is a rapid approach for developing propagation protocols, can be useful for the massive production of these endangered *Cattleya* species. For these reasons, the aim of this study was to evaluate the effects of two culture media MS and KC, in combination with different concentrations of plant growth regulators NAA and GA₃, on *in vitro* germination and development of *Cattleya mendelii* and *C. quadricolor*, to establish a protocol for the *in vitro* propagation of both species to aid in their conservation.

MATERIALS AND METHODS

PLANT MATERIAL AND ESTABLISHMENT OF *IN VITRO* CULTURE

Closed capsules of *Cattleya mendelii* and *C. quadricolor* were obtained from the commercial nursery (Las Orquídeas, San Antonio del Tequendama, Colombia, 4° 34' 17" N, 74° 18' 45" W; 2000 masl), where the parental plants had been selected and cross pollinated by hand for this study. When the capsules started to turn yellow, they were harvested with sterile garden shears and transported to the *in vitro* culture laboratory of the Fundación Zoológico Santa Cruz, where they were kept during 1 day in the laminar flow hood until the start of the experiment.

While in the laminar flow hood, the capsules were disinfected by immersion in 90% ethanol (v/v) for five minutes, rinsed with sterile distilled water, and submerged in 5% sodium hypochlorite for 20 min, and rinsed with sterile distilled water. Each capsule was longitudinally dissected in a sterile Petri dish, where approximately half of the seeds were suspended in 70 mL of sterile distilled water. After shaking the seed suspension with a vortex, an aliquot of 1

mL, which contained ca. 50 seeds were placed uniformly in glass bottles containing the experimental culture medium.

EXPERIMENTAL TREATMENTS

Seed germination and seedling development were evaluated by planting the seeds in either KC (Knudson 1946) or MS (Murashige & Skoog 1962) culture media enriched with sucrose (30 g/L) and experimentally amended with various combinations of giberelic acid (GA₃) and naphthaleneacetic acid (NAA): 0/0; 0.5/1.5; 1.0/1.0; 1.5/0.5 μM respectively. For each experimental treatment, 25 mL of growth medium were added to a glass bottle of 120 mL in volume, in which the seeds were planted as described above.

The glass bottles were placed in a tissue culture room at 22 ± 2 °C and a photoperiod of 12 h. Plant development was evaluated every 30 days and the number of individuals at the various stages were recorded as follows: germination (defined as the stage when the embryo emerges from the integument) at 30 days, protocorm-like bodies (the embryo is completely released from integument) at 60 days, shoots (the protocorm is differentiated) at 90 days, and seedlings (emergence of leaf blades, rhizomes) were recorded at 120 days.

After 120 days of culture the number of roots was counted and leaf and root length were measured with a vernier caliper (readable to 0.02 millimeters). The number of leaves was not counted because for these species the maximum number of leaves is fixed to one or two and does not respond to treatments at 120 days of *in vitro* culture (unpublished observations).

DATA ANALYSIS

Data were analyzed under a factorial arrangement according to a fully randomized design. For each species, the stage of development and seedling growth were compared among medium and hormone treatment with two way ANOVAs followed by a *post hoc* Holm Sidak test (at $p < 0.05$). All analyses were conducted with Sigmasat 3.5 (Systat®, Richmond California, USA). Data are shown as means ± 1 S.E. for 10 glass bottles containing 50 seeds each that were prepared as described above.

RESULTS

CATTLEYA MENDELII

For *C. mendelii* the MS medium promoted a higher seed germination than the KC medium (Table I; Fig. 1a). At 30 days of incubation, the highest germination rate of 95.2 ± 0.8% was observed for seeds grown on MS medium amended with 1.5 μM of GA₃ and 0.5 μM of NAA, while the lowest germination of 41.7 ± 1.2% was recorded for seeds incubated with no growth regulator enrichment in KC medium (Fig. 1a).

At 60 days of incubation, *C. mendelii* had the highest protocorm development when grown on MS than on KC (Table I; Fig. 1b). In particular, the highest survival at this stage of development, $94.6 \pm 0.8\%$, was observed for plants growing in MS amended with $1.0 \mu\text{M GA}_3$ and $1.0 \mu\text{M NAA}$, while the lowest survival was recorded for plants incubated in KC without growth regulator enrichment.

At 90 days of incubation, a significant interaction was found between the medium and the growth regulator amendment for shoot yield of both orchids (Table I; Fig. 1c). In particular, for *C. mendelii* the highest percentage of surviving plants with shoots was $97.1 \pm 0.6\%$ observed for individuals growing in MS amended with $0.5 \mu\text{M GA}_3$ and $1.5 \mu\text{M NAA}$.

At 120 days of incubation a greater seedling survival was observed for *C. mendelii* growing on MS than on KC (Table I; Fig. 1d). In particular, the highest seedling survival of $95.9 \pm 0.9\%$ was observed under incubation with MS amended with $0.5 \mu\text{M GA}_3$ and $1.5 \mu\text{M NAA}$, while the lowest survival of $60.3 \pm 1.0\%$ was recorded under incubation with KC with no growth regulator amendment.

Leaf length for *C. mendelii* was affected by the culture medium utilized (Table I; Fig. 2a). For individuals incubated in MS amended with $0.5 \mu\text{M GA}_3$ and $1.5 \mu\text{M NAA}$, a leaf length of 11.1 ± 0.5 mm and the presence of 4.1 ± 0.4 roots per plant with an average length of 9.6 ± 0.5 mm, were consistently higher than for plants incubated with other culture media (Fig. 2b, c). Contrasting was the case of plants incubated with no hormonal enrichment in MS whose leaf length and root number and length only amounted to 5.0, 2.4, and 4.4 mm respectively.

CATTELEYA QUADRICOLOR

C. quadricolor showed significant differences on seed germination among the growth media (Table I; Fig. 1e). At 30 days of incubation, the highest germination of $84.3 \pm 1.1\%$ for seeds incubated in KC and $96.4 \pm 0.6\%$ in MS was observed for seeds incubated with $1.5 \mu\text{M GA}_3$ and $0.5 \mu\text{M NAA}$. The lowest germination of $54.2 \pm 1.1\%$ in KC and $59.3 \pm 1.0\%$ in MS medium was recorded for seeds incubated with no growth regulator amendment.

Statistical differences on protocorm survival were observed for *C. quadricolor* plants growing on either incubation medium (Table I; Fig. 1f). The highest protocorm survival of $95.7 \pm 0.7\%$ for plants grown in MS enriched with $0.5 \mu\text{M GA}_3$ and $1.5 \mu\text{M NAA}$. The lowest protocorm survival for *C. quadricolor* was recorded for plants grown on either medium without growth regulator amended.

The lowest yield of shoots for *C. mendelii* at 90 days of incubation was observed for plants grown in KC without growth regulators yielded $52.0 \pm 0.8\%$. For *C. quadricolor*, the highest yield of $95.6 \pm 1.1\%$ was observed for plants incubated with MS amended with $0.5 \mu\text{M GA}_3$ and $1.5 \mu\text{M NAA}$. The lowest yield of shoots of $62.9 \pm 1.1\%$ was

observed in MS amended with $1.5 \mu\text{M GA}_3$ and $0.5 \mu\text{M NAA}$.

Significant interaction was found between culture medium and growth regulator amendment for seedling survival of *C. quadricolor* (Table I; Fig. 1h). The highest seedling survival of $94.4 \pm 1.0\%$ was recorded for plants incubated in MS enriched with $0.5 \mu\text{M GA}_3$ and $1.5 \mu\text{M NAA}$, while the lowest seedling survival of $64.5 \pm 3.5\%$ was observed for plants incubated in KC with no growth regulator amendment.

Seedling growth for this species was enhanced by the incubation in MS (Table I). Leaf length ranged from 5.1 ± 0.41 mm for plants incubated in either medium without hormonal amended to 10.2 ± 0.4 mm for seedlings grown in MS amended with $0.5 \mu\text{M GA}_3$ and $1.5 \mu\text{M NAA}$ (Fig. 2d). The highest count of 4.2 ± 0.3 roots per plant was found for plants incubated with MS amended with $0.5 \mu\text{M GA}_3$ and $1.5 \mu\text{M NAA}$ and KC amended with $1.0 \mu\text{M GA}_3$ and $1.0 \mu\text{M NAA}$ (Fig. 2e). The lowest root number of 2.0 ± 0.3 was found for plants incubated in MS medium without hormonal amendment. Root length for seedlings of *C. quadricolor* averaged 8.9 ± 0.3 mm in KC and 9.2 ± 0.4 mm in MS medium with hormonal amended (Fig. 2f). It decreased by 33.7% for seedlings incubated in MS medium without hormonal amendment and by 51.7% for seedlings incubated in KC medium without hormonal amended.

DISCUSSION

While a high germination percentage was observed for both *Cattleya mendelii* and *C. quadricolor* under all treatments, MS was the best medium for this and all subsequent developmental stages. For germination, MS was best in combination with $1.5 \mu\text{M GA}_3$ and $0.5 \mu\text{M NAA}$, while for the remaining stages MS amended with $0.5 \mu\text{M GA}_3$ and $1.5 \mu\text{M NAA}$ yielded higher survival. An exception was protocorm development for *C. mendelii*, whose largest number was observed for plants incubated in $1.0 \mu\text{M GA}_3$ and $1.0 \mu\text{M NAA}$ in MS medium. Additionally, KC and MS were equally effective for leaf and root growth, as well as for root number of *C. mendelii* when amended with $0.5 \mu\text{M GA}_3$ and $1.5 \mu\text{M NAA}$ for *C. quadricolor*. KC and MS media amended with $0.5 \mu\text{M GA}_3$ and $1.5 \mu\text{M NAA}$, showed differences in root number and leaf length.

Ammonium salts yield better *in vitro* growth than nitrates (Raghavan & Torrey 1964, Hew & Yong 2004). Seeds respond directly to the concentration of ammonium nitrate in the medium. For instance, when seeds germinate in a medium lacking NH_4^+ , small protocorms are formed and further growth is suppressed (Raghavan & Torrey 1964, Kauth *et al.* 2008). Here, MS was a better culture medium for both species of *Cattleya* because its composition that includes ammonium nitrate provides the same proportion

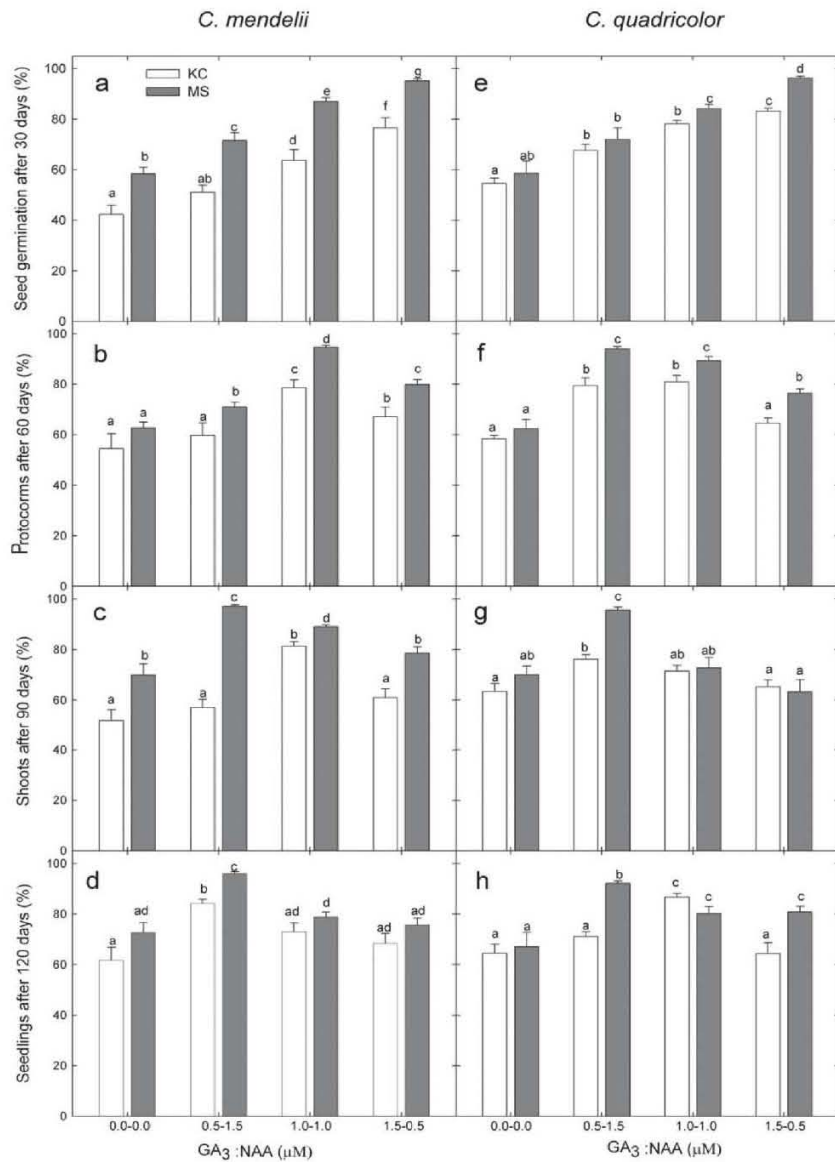


FIGURE 1. Survival for *Cattleya mendelii* (a-d) and *C. quadricolor* (e-h) at various developmental stages growing on KC (Knudson 1946; white bars) and MS (Murashige & Skoog 1962; grey bars) amended with various concentrations of GA₃ and NAA. Seeds that germinated (a, e) were counted at 30 days of incubation, while protocorms (b, f), were counted at 60 days of incubation. Shoots (c, g) were counted at 90 days of incubation and the ensuing seedlings (d, h) at 120 days of culture. Data are shown as mean ± S.E. (n = 10 glass bottles containing ca. 50 seeds each). For each panel, different letters indicate a statistical difference ($p < 0.05$) from Holm-Sidak test following 2-way ANOVAs.

FIGURA 1. Sobrevivencia de *Cattleya mendelii* (a-d) y *C. quadricolor* (e-h) en distintas etapas del desarrollo incubadas en los medios KC (Knudson 1946; barras blancas) y MS (Murashige & Skoog 1962; barras grises) enriquecidos con varias concentraciones de GA₃ y NAA. Las semillas que germinaron (a, e) se contaron a los 30 días de incubación, mientras que los protocormos (b, f) se contaron a los 60 días de incubación. Los brotes (c, g) se contaron a los 90 días de incubación y las plántulas subsecuentes (d, h) a los 120 días de incubación. Se muestran los datos como promedio ± E.E. (n = 10 frascos de cultivo con ca. 50 semillas cada uno). Para cada panel, letras distintas indican diferencias estadísticas ($p < 0.05$) de un ANOVA de 2 vías seguido de una prueba de Holm-Sidak.

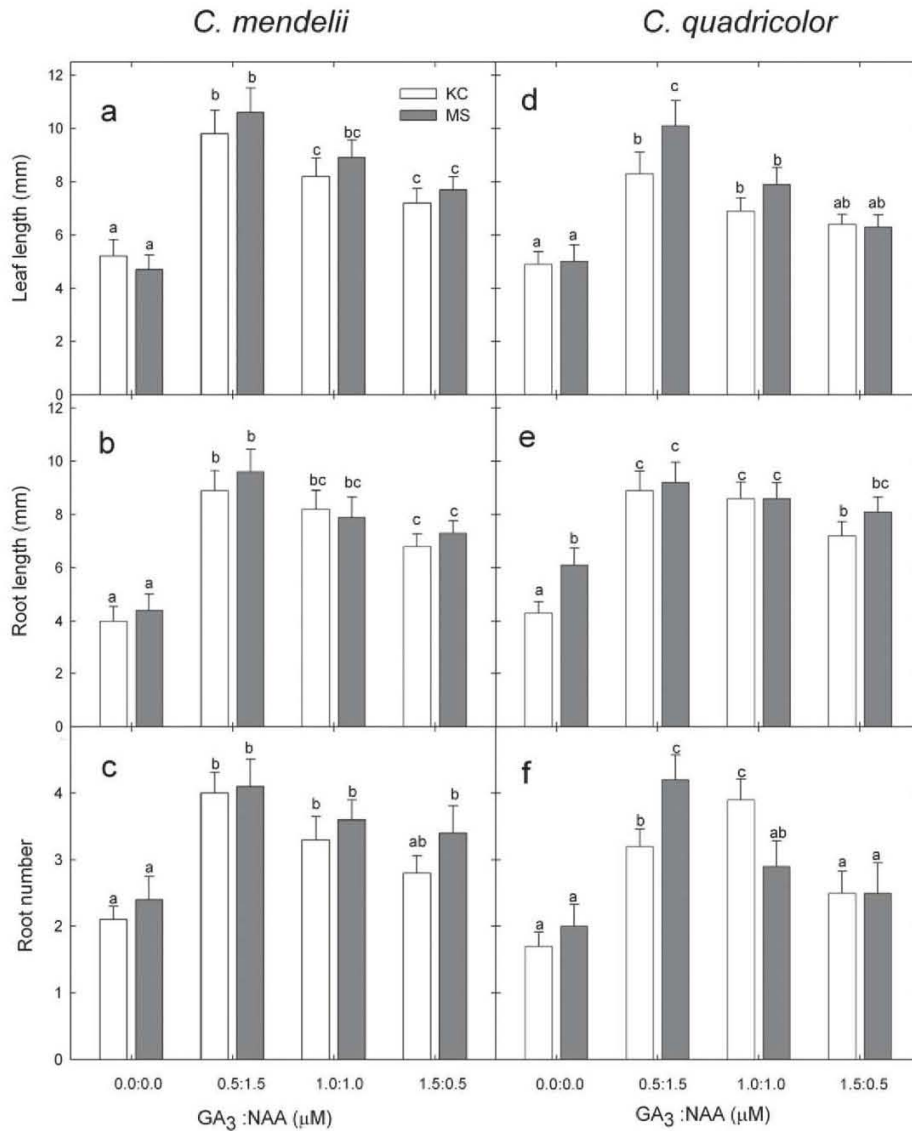


FIGURE 2. Seedling growth for *C. mendelii* (a-c) and *C. quadricolor* (d-f). Leaf length (a, d), root number (b, e) and root length (c, f) were determined at 120 days of incubation for seedlings growing KC (Knudson 1946; white bars) and MS (Murashige & Skoog 1962; grey bars) enriched with various concentrations of GA₃ and NAA. Data are shown as means ± S.E. (n = 10 glass bottles containing ca. 50 seeds each). For each panel, different letters indicate a statistical difference ($p < 0.05$) from Holm-Sidak test following 2-way ANOVAs.

FIGURA 2. Crecimiento de plántulas de *C. mendelii* (a-c) y *C. quadricolor* (d-f). La longitud de la hoja (a, d), número (b, e) y longitud (c, f) de raíces se determinaron a los 120 días de incubación para plántulas creciendo en los medios KC (Knudson 1946; barras blancas) y MS (Murashige & Skoog 1962; barras grises) enriquecidos con distintas concentraciones de GA₃ y NAA. Los datos se muestran como promedio ± E.E. (n = 10 frascos de cultivo con ca. 50 semillas cada uno). Para cada panel, letras distintas indican diferencias estadísticas ($p < 0.05$) de un ANOVA de dos vías seguido de una prueba de Holm-Sidak.

TABLE I. Two way ANOVAs of seedling growth and plant development for *Cattleya mendelii* and *C. quadricolor*.

TABLA I. ANOVA de dos vías del crecimiento de plántulas y desarrollo de *Cattleya mendelii* y *C. quadricolor*.

<i>Cattleya mendelii</i>																					
	SEED GERMINATION			PROTOCORMS			SHOOTS			SEEDLINGS			LEAF LENGTH			ROOT LENGTH			ROOT NUMBER		
	df	F	P	df	F	P	df	F	P	df	F	P	df	F	P	df	F	P	df	F	P
Medium	1	83.2	<0.001	1	23.8	<0.001	1	103	<0.001	1	14.9	<0.001	1	1.36	0.24	1	0.88	0.35	1	2.03	0.15
Growth regulator	3	52.7	<0.001	3	23.5	<0.001	3	23.8	<0.001	3	18.2	<0.001	3	47.2	<0.001	3	38.9	<0.001	3	10.9	<0.001
Medium × regulator	3	0.50	0.68	3	0.42	0.73	3	10.7	<0.001	3	0.38	0.76	3	0.86	0.86	3	0.39	0.75	3	0.20	0.89
<i>Cattleya quadricolor</i>																					
	SEED GERMINATION			PROTOCORMS			SHOOTS			SEEDLINGS			LEAF LENGTH			ROOT LENGTH			ROOT NUMBER		
	df	F	P	df	F	P	df	F	P	df	F	P	df	F	P	df	F	P	df	F	P
Medium	1	12.7	<0.001	1	37.3	<0.001	1	8.33	0.005	1	13.9	<0.001	1	4.89	0.03	1	8.36	0.05	1	0.10	0.751
Growth regulator	3	55.4	<0.001	3	62.2	<0.001	3	19.2	<0.001	3	13.2	<0.001	3	32.0	<0.001	3	43.9	<0.001	3	12.8	<0.001
Medium × regulator	3	1.22	0.30	3	2.04	0.11	3	4.62	0.005	3	7.21	<0.001	3	1.91	0.13	3	2.34	0.08	3	3.09	0.03

of ammonium and nitrate to seeds (Murashige & Skoog 1962). In contrast, the KC medium has a higher proportion of nitrate than ammonium (Knudson 1946). In consequence, considering that nitrogen uptake by roots is primarily mediated by the enzyme nitrate reductase, which assimilates nitrates the plants should have responded better to KC. However, the enzyme nitrate reductase is not expressed in orchid tissues until 60 days of *in vitro* germination making the MS medium better than KC medium for plants in early stages of development (Hew & Yong 2004).

Higher GA₃ to NAA ratios improved germination for both species incubated in either medium, because the direct effect on *in vitro* germination by the different GA₃ concentrations (Santos-Hernández *et al.* 2005). This phytohormone activates the α -amylase that mediates sugar absorption from the culture medium (Held & Piechulla, 2010). Thus an ammonium enriched medium such as MS enriched with high concentrations of GA₃ can yield very high germination responses as was the case in present work.

The later developmental stages (protocorms, shoots and seedlings) for both orchids, was generally enhanced by a higher proportion of NAA to GA₃. This is similar to the developmental responses of *Dendrobium* species, for which the number of shoots increases in response to the concentration of NAA (Parvin *et al.* 2009), and for *Grammatophyllum speciosum* cultivated in a medium enriched with NAA (Sopalun *et al.* 2010).

A higher proportion of NAA than GA₃ produced more and longer roots and longer leaves for both species grown in MS. For another Neotropical orchid, *Laelia speciosa*, GA₃ produces longer seedlings (Ávila-Díaz *et al.* 2009). Here, high concentrations of GA₃ resulted in shorter leaves and roots than those of plants incubated in medium without this phytohormone.

CONCLUSIONS

The combination of growth regulators and culture media utilized for the *in vitro* production of *Cattleya mendelii* and *C. quadricolor* from seeds was highly successful in this study, yielding vast number of seedlings of these endangered Colombian orchids. This method can be useful for producing plants for possible reintroductions into natural habitats and for commercial purposes in different markets, in order to decrease the extractive pressure facing natural populations.

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